

## TUMOR NECROSIS FACTOR-GAMMA

This application claims benefit under 35 U.S.C. § 119(e) of the filing date of copending U.S. Provisional Application Serial No. 60/074,047, filed on February 9, 1998. Further, this application claims benefit under 35 U.S.C. § 120 as a continuation-in-part of U.S. Application Serial No. 09/131,237, filed on August 7, 1998, which is a continuation-in-part of U.S. Application Serial No. 09/005,020, filed on January 9, 1998, which is a continuation-in-part of U.S. Application Serial No. 08/461,246, filed on June 5, 1995, which is a continuation-in-part of International Application Serial No. PCT/US94/12880, filed on November 7, 1994. *Each of the five aforementioned applications* are hereby incorporated by reference in their entireties.

### Field of the Invention

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention has been identified as a new member of the tumor necrosis factor family and is hereinafter referred to as "TNF-gamma-alpha". The invention also relates to a protein encoded by a splice variant of the gene encoding TNF-gamma-alpha which is hereinafter referred to as "TNF-gamma-beta". The invention also relates to inhibiting the action of such polypeptides.

### Background of the Invention

Human tumor necrosis factors-a (TNF-a) and b (TNF-b or lymphotoxin) are related members of a broad class of polypeptide mediators, which includes the interferons, interleukins and growth factors, collectively called cytokines (Beutler, B. and Cerami, A., Annu. Rev. Immunol., 7:625-655 (1989)).

Tumor necrosis factor (TNF-a and TNF-b) was originally discovered as a result of its anti-tumor activity, however, now it is recognized as a pleiotropic cytokine playing important roles in immune regulation and inflammation. To date, there are eight known members of the TNF-related cytokine family, *TNF-alpha, TNF-beta, lymphotoxin (LT)-alpha, LT-beta*, and ligands for the Fas, CD30, CD27, CD40 and 4-1BB receptors. These proteins have conserved C-terminal sequences and variable N-terminal sequences which are often used as membrane anchors, with the exception of

~~TNF-beta. Both TNF-alpha and TNF-beta~~  
 a ~~TNF-b. Both TNF-a and TNF-b~~ function as homotrimers when they bind to TNF receptors.

TNF is produced by a number of cell types, including monocytes, fibroblasts, T-cells, natural killer (NK) cells and predominately by activated macrophages. <sup>TNF-alpha</sup>

5 a has been reported to have a role in the rapid necrosis of tumors, immunostimulation, autoimmune disease, graft rejection, resistance to parasites, producing an anti-viral response, septic shock, growth regulation, vascular endothelium effects and metabolic

a effects. <sup>TNF-alpha also triggers</sup> TNF-a also triggers endothelial cells to secrete various factors, including PAI-

10 a 1, IL-1, <sup>TNF-alpha</sup> GM-CSF and IL-6 to promote cell proliferation. In addition, <sup>TNF-alpha</sup> TNF-a up-regulates various cell adhesion molecules such as E-Selectin, ICAM-1 and VCAM-1.

a <sup>TNF-alpha</sup> TNF-a and Fas ligand have also been shown to induce programmed cell death.

The first step in the induction of the various cellular responses mediated by TNF or LT is their binding to specific cell surface receptors. Two distinct TNF receptors of approximately 55-KDa (TNF-R1) and 75-KDa (TNF-R2) have been identified (Hohman, H.P. et al., J. Biol. Chem., 264:14927-14934 (1989)), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher, H. et al., Cell, 61:351 (1990)). Both TNF-Rs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions.

20 The endothelium, which under physiological conditions is mostly a quiescent tissue (Denekamp, J. *Cancer Metas. Rev.* 9:267-282 (1990)), plays an essential role in the maintenance of vascular homeostasis and permeability. Endothelial cells are actively involved in inflammation, cell adhesion, coagulation, thrombosis, fibrinolysis, and angiogenesis. During angiogenesis, endothelial cells proliferate, 25 invade into stroma, migrate toward the source of an angiogenesis stimulus, such as cancer cells, interact with perivascular cells and stromal cells, and eventually, form capillary vessels linking the tumor tissue to the circulatory system (Folkman, J. *Nature Med.* 1:27-31 (1995)). Although the complex mechanism that regulates angiogenesis is yet to be fully understood, it is becoming clear that the initiation or 30 termination of the process is a result of a balance between positive and negative factors.

A number of angiogenic factors, often markedly upregulated in tumor tissues, have been described. These include several members of the fibroblast growth factor (FGF) family, such as FGF-1, FGF-2, and those of the vascular endothelial cell 35 growth factor (VEGF) family and the receptors for all of these molecules (Gimenez-Gallego, G, et al., *Science* 230:1385-1388 (1985); Schweigerer, L., et al., *Nature* 325:257-259 (1987); Leung, D. W., et al., *Science* 246:1306-1309 (1989);

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## Summary of the Invention

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The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding at least a portion of the TNF-gamma-alpha polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 or the complete amino acid sequence encoded by the cDNA clone HUVEO91 deposited as plasmid DNA as ATCC Deposit Number 75927 at the American Type Culture Collection ("ATCC") on October 26, 1994. The ATCC is located at 10801 University Boulevard, Manassas, VA 20110-2209, USA. The nucleotide sequence determined by sequencing the deposited TNF-gamma-alpha clone, which is shown in Figures 1A and 1B (SEQ ID NO:1), contains an open reading frame encoding a complete polypeptide of 174 amino acid residues, including an initiation codon encoding an N-terminal methionine at nucleotide positions 783-785, and a predicted molecular weight of about 20,132 Da.

*Sub C* The present invention also provides isolated nucleic acid molecules comprising a polynucleotide encoding at least a portion of the TNF-gamma-beta polypeptide having the complete amino acid sequence shown in SEQ ID NO:20 or the complete amino acid sequence encoded by the cDNA clone HEMCZ51 deposited as plasmid DNA as ATCC Deposit Number 203055 on July 9, 1998. The nucleotide sequence determined by sequencing the deposited TNF-gamma-beta clone, which is shown in Figures 20A and B (SEQ ID NO:20), contains an open reading frame encoding a complete polypeptide of 251 amino acid residues, including an initiation codon encoding an N-terminal methionine at nucleotide positions 1-3, and a predicted molecular weight of about 28,089 Da.

Thus, in one embodiment the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding the TNF-gamma-alpha polypeptide having the complete amino acid sequence in SEQ ID NO:2 (i.e., positions -27 to 147 of SEQ ID NO:2, (b) a nucleotide sequence encoding the TNF-gamma-alpha polypeptide having the complete amino acid sequence in SEQ ID NO:2 excepting the N-terminal methionine (i.e., positions -26 to 147 of SEQ ID NO:2); (c) a nucleotide sequence encoding the mature TNF-gamma-alpha polypeptide having the amino acid sequence in SEQ ID NO:2 shown as positions 1 to 147 of SEQ ID NO:2; (d) a nucleotide sequence encoding the TNF-gamma-alpha polypeptide having the complete amino acid sequence encoded by the cDNA clone HUVEO91 contained in ATCC Deposit No. 75927; (e) a nucleotide sequence encoding the TNF-gamma-alpha polypeptide having the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone HUVEO91 contained in ATCC Deposit No. 75927; (f) a nucleotide sequence encoding the mature TNF-gamma-alpha

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polypeptide having the amino acid sequence encoded by the cDNA clone HUVEO91 contained in ATCC Deposit No. 75927; and (g) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e) or (f), above.

*Sub 2*  
 5 In another embodiment, the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding the TNF-gamma-beta polypeptide having the complete amino acid sequence in SEQ ID NO:20 (i.e., positions 1 to 251 of SEQ ID NO:20); (b) a nucleotide sequence encoding the ~~TNF-gamma-alpha~~ <sup>TNF-gamma-beta</sup> polypeptide having the complete amino acid sequence in SEQ ID  
 10 NO:20 excepting the N-terminal methionine (i.e., positions 2 to 251 of SEQ ID NO:20); (c) a nucleotide sequence encoding the mature ~~TNF-gamma-alpha~~ <sup>TNF-gamma-beta</sup> polypeptide having the amino acid sequence in SEQ ID NO:20 shown as positions 62 to 251 of SEQ ID NO:20; (d) a nucleotide sequence encoding the ~~TNF-gamma-alpha~~ <sup>TNF-gamma-beta</sup> polypeptide having the complete amino acid encoded by the cDNA clone HUVEO91  
 15 contained in ATCC Deposit No. 203055; (e) a nucleotide sequence encoding the ~~TNF-gamma-alpha~~ <sup>TNF-gamma-beta</sup> polypeptide having the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone HUVEO91 contained in ATCC Deposit No. 203055; (f) a nucleotide sequence encoding the mature TNF-gamma-beta polypeptide having the amino acid sequence encoded by the cDNA clone HUVEO91  
 20 contained in ATCC Deposit No. 203055; and (g) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e) or (f), above.

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% identical,  
 25 and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f) or (g), above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide in (a), (b), (c), (d), (e), (f) or (g), above, a fragment thereof (such as, for example, fragments described herein), or the complementary strand thereto. This  
 30 polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues. An additional nucleic acid embodiment of the invention relates to an isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion (i.e., a  
 35 fragment) of a TNF-gamma polypeptide having an amino acid sequence in (a), (b), (c), (d), (e) or (f), above. A further embodiment of the invention relates to an isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid

sequence of a TNF-gamma polypeptide having an amino acid sequence which contains at least one amino acid substitution, but not more than 50 amino acid substitutions, even more preferably, not more than 40 amino acid substitutions, still more preferably, not more than 30 amino acid substitutions, and still even more preferably, not more than 20 amino acid substitutions. Of course, in order of ever-increasing preference, it is highly preferable for a polynucleotide which encodes the amino acid sequence of a TNF-gamma polypeptide to have an amino acid sequence which contains not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid substitutions. Conservative substitutions are preferable.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of TNF-gamma polypeptides or peptides by recombinant techniques.

In accordance with a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a human TNF-gamma nucleic acid sequence, under conditions promoting expression of said protein and subsequent recovery of said protein.

The invention further provides an isolated TNF-gamma polypeptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of the full-length TNF-gamma-alpha polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 (i.e., positions -27 to 147 of SEQ ID NO:2); (b) the amino acid sequence of the full-length TNF-gamma-alpha polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 excepting the N-terminal methionine (i.e., positions -26 to 147 of SEQ ID NO:2); (c) the amino acid sequence of the predicted mature TNF-gamma-alpha polypeptide having the amino acid sequence at positions 1-147 in SEQ ID NO:2; (d) the complete amino acid sequence encoded by the cDNA clone HUVEO91 contained in the ATCC Deposit No. 75927; (e) the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone HUVEO91 contained in the ATCC Deposit No. 75927; (f) the complete amino acid sequence of the predicted mature TNF-gamma polypeptide encoded by the cDNA clone HUVEO91 contained in the ATCC Deposit No. 75927; and (g) fragments of the polypeptide of (a), (b), (c), (d), (e), or (f). The polypeptides of the present invention also include polypeptides having an amino acid sequence at least 80% identical, more preferably at least 90% identical, and still more preferably 95%, 96%, 97%, 98% or 99% identical to those described in

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B<sub>15</sub>

B

20 B

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with at least six or seven, preferably at least nine, and more preferably at least about 30 amino acids to about 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire amino acid sequence of a polypeptide of the invention described above also are included in the invention.

5 A further embodiment of the invention relates to a polypeptide which comprises the amino acid sequence of a TNF-gamma polypeptide having an amino acid sequence which contains at least one amino acid substitution, but not more than 50 amino acid substitutions, even more preferably, not more than 40 amino acid substitutions, still more preferably, not more than 30 amino acid substitutions, and  
10 still even more preferably, not more than 20 amino acid substitutions. Of course, in order of ever-increasing preference, it is highly preferable for a peptide or polypeptide to have an amino acid sequence which comprises the amino acid sequence of a TNF-gamma polypeptide, which contains at least one, but not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid substitutions. In specific embodiments, the number of  
15 additions, substitutions, and/or deletions in the amino acid sequence of Figures 1A and 1B, Figures 20 A and B, or fragments thereof (e.g., the extracellular domain and/or other fragments described herein), is 1-5, 5-10, 5-25, 5-50, 10-50 or 50-150, conservative amino acid substitutions are preferable.

In another embodiment, the invention provides an isolated antibody that binds  
20 specifically to a TNF-gamma polypeptide having an amino acid sequence described above. The invention further provides methods for isolating antibodies that bind specifically to a TNF-gamma polypeptide having an amino acid sequence as described herein. Such antibodies are useful diagnostically or therapeutically as described below.

25 In accordance with yet a further aspect of the present invention, there is provided a process for utilizing polynucleotides and/or polypeptides of the invention to screen for agonists and antagonists, and for therapeutic purposes, which include, but are not limited to, wound healing, to inhibit tumor proliferation, to provide resistance to parasites, bacteria and viruses, to induce inflammatory activities, to  
30 induce proliferation of endothelial cells and certain hematopoietic cells, to treat restenosis and to prevent certain autoimmune diseases.

In accordance with yet a further aspect of the present invention, there are also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to human TNF-gamma sequences.

35 In accordance with another aspect of the present invention, there are provided TNF-gamma agonists which mimic TNF-gamma and binds to the TNF-gamma receptors to elicit TNF-gamma type responses.

5 In accordance with still another aspect of the present invention, there are provided diagnostic assays for detecting diseases related to the under-expression and over-expression of the TNF-gamma polypeptide and nucleic acid sequences encoding such polypeptide.

In yet another aspect, the TNF-gamma may bind to a cell surface protein which also functions as a viral receptor or coreceptor. Thus, TNF-gamma, or agonists or antagonists thereof, may be used to regulate viral infectivity at the level of viral binding or interaction with the TNF-gamma receptor or coreceptor or during the process of viral internalization or entry into the cell.

20            These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figures 1A and 1B illustrate the cDNA (SEQ ID NO:1) and corresponding deduced amino acid sequence (SEQ ID NO:2) of the polypeptide of TNF-gamma-alpha of the present invention. The initial 27 amino acids (underlined) are the putative leader sequence. The standard one-letter abbreviations for amino acids are used. Potential asparagine-linked glycosylation sites are marked in Figures 1A and 1B with a bolded asparagine symbol (N) in the TNF-gamma-alpha amino acid sequence and a bolded pound sign (#) above the first nucleotide encoding that asparagine residue in the TNF-gamma-alpha nucleotide sequence. Potential N-linked glycosylation sequences are found at the following locations in the TNF-gamma-alpha amino acid sequence: N-29 through N-32 (N-29, Y-30, T-31, N-32) and N-125 through D-128 (N-125, V-126, S-127, D-128). Potential Protein Kinase C (PKC) phosphorylation sites are also marked in Figures 1A and 1B with a bolded threonine

symbol (T) in the TNF-gamma-alpha amino acid sequence and an asterisk (\*) above the first nucleotide encoding that threonine residue in the TNF-gamma-alpha nucleotide sequence. Potential PKC phosphorylation sequences are found at the following locations in the TNF-gamma-alpha amino acid sequence: T-32 through K-34 (T-32, N-33, K-34) and T-50 through R-52 (T-50, F-51, R-52). Potential Casein Kinase II (CK2) phosphorylation sites are also marked in Figures 1A and 1B with a bolded serine or threonine symbol (S or T) in the TNF-gamma-alpha amino acid sequence and an asterisk (\*) above the first nucleotide encoding the appropriate serine or threonine residue in the TNF-gamma-alpha nucleotide sequence. Potential CK2 phosphorylation sequences are found at the following locations in the TNF-gamma-alpha amino acid sequence: S-83 through E-86 (S-83, Y-84, P-85, E-86); S-96 through E-99 (S-96, V-97, C-98, E-99); S-115 through E-118 (S-115, L-116, Q-117, E-118); S-130 through D-133 (S-130, L-131, V-132, D-133); and T-135 through D-138 (T-135, K-136, E-137, D-138). Potential myristylation sites are also marked in Figures 1A and 1B with a double underline in the TNF-gamma-alpha amino acid sequence. Potential myristylation sequences are found at the following locations in the TNF-gamma-alpha amino acid sequence: G-20 through K-25 (G-20, L-21, A-22, F-23, T-24, K-25) and G-111 through L-116 (G-111, A-112, M-113, F-114, S-115, L-116).

Sub C3 Figures 2A-C illustrate an amino acid sequence alignment between TNF-gamma-alpha (SEQ ID NO:2) and other members of the TNF family including human TNF-alpha (GenBank No. Z15026; SEQ ID NO:3), human TNF-beta (GenBank No. Z15026; SEQ ID NO:4), human lymphotoxin-beta (LTbeta; GenBank No. L11016; SEQ ID NO:5), and human Fas Ligand (FASL; GenBank No. U11821; SEQ ID NO:6). TNF-gamma contains the conserved amino acid residues of the TNF family as shown by the boxed and shaded areas. The aligned molecules are presented in their entirety as Figures 2A, 2B, and 2C.

Figure 3A is an RNA blot analysis showing the human tissues where TNF-gamma is expressed. RNA from the tissues indicated were probed with labeled TNF-gamma cDNA. TNF-gamma-alpha mRNA exists predominantly in the kidney since Figure 3A shows a distinct band. Other lanes seem to show strong hybridization, however, these are actually non-specific smears.

Figure 3B is an RNA blot analysis showing that TNF-gamma is expressed predominantly in HUVEC cells (human umbilical vein endothelial cells; lane 9). Lane 6 and lane 8 are non-specific smears. RNA from the cell lines indicated were probed with labeled TNF-gamma-alpha cDNA. Lane 1 is CAMA1 (breast cancer); lane 2 AN3CA (uterine cancer); lane 3, SK.UT.1 (uterine cancer); lane 4, MG63

(osteoblastoma); lane 5, HOS (osteoblastoma); lane 6, MCF7 (breast cancer); lane 7, OVCAR-3 (ovarian cancer); lane 8, CAOV-3 (ovarian cancer); lane 9, HUVEC; lane 10, AOSMIC (smooth muscle); lane 11, foreskin fibroblast.

Figure 4 illustrates the relative expression of TNF-gamma in proliferating or quiescent endothelial cells. The TNF-gamma mRNA levels in cultured HUVEC cells were determined by Northern blotting analysis. Identical amounts of total RNA (15  $\mu$ g) were loaded on each lane, as indicated by the <sup>intensity of beta-actin</sup> ~~intensity of b-actin~~. The signal which corresponds to TNF-gamma is designated "VEGI". Total RNA was prepared at the indicated time point (days post-seeding). The number of cells in each culture flask was determined simultaneously. The experiment was carried out in duplicate. Cells were seeded at 125,00 cells per flask (T-25).

Figure 5 is a photograph of a polyacrylamide gel electrophoresis analysis of TNF-gamma protein. TNF-gamma was produced by bacterial expression and purified as described in Example 1.

Figure 6 is a photograph of a gel showing the relative purity and mobility of baculovirus-expressed TNF-gamma. The expression and purification of TNF-gamma using the baculovirus system is described in Example 2.

Figure 7A consists of photographs of WEHI 164 cells which are untreated (Figure 7Aa) and after exposure to <sup>TNF-alpha</sup> ~~TNF-a~~ (Figure 7Ab), TNF-gamma (Figure 7Ac), and <sup>TNF-beta</sup> ~~TNF-b~~ (Figure 7Ad). Cells which have an elongated non-round morphology have been lysed. The various TNF molecules were added at a concentration of approximately 0.5  $\mu$ g/ml. Photographs were taken 72 hours after addition of the various TNF molecules.

Figure 7B illustrates the ability of TNF-gamma (Figure 7Bc) in comparison to <sup>TNF-alpha (Figure 7Ba) and TNF-beta (Figure 7Bb)</sup> ~~TNF-a (Figure 7Ba) and TNF-b (Figure 7Bb)~~ to inhibit WEHI 164 cell growth.

Figure 8 illustrates the ability of recombinant <sup>TNF-alpha (Figure 8B), TNF-beta</sup> ~~TNF-a (Figure 8B), TNF-b~~ (Figure 8D), and TNF-gamma (Figure 8C) to induce morphological change in L929 cells with respect to untreated L929 cells (Figure 8A). The morphology change is indicated by dark round cells. Cells were treated with the various recombinant TNF molecules (produced in *E. coli*) at approximately 0.5  $\mu$ g/ml. The photographs were taken 72 hours after the addition of the various TNF molecules. The morphology change indicates that the cells have been killed.

Figure 9 is a graphical illustration of the effect of TNF-gamma (Figure 9C), <sup>TNF-alpha (Figure 9A), and TNF-beta (Figure 9B)</sup> ~~TNF-a (Figure 9A), and TNF-b (Figure 9B)~~ on venous endothelial cells. Cell proliferation after venous endothelial cells were treated with commercially available <sup>TNF-alpha and TNF-beta</sup> ~~TNF-a and TNF-b~~ and *E. coli* produced TNF-gamma was quantified using an MTS assay.

Figure 10 shows the effect of TNF-gamma on the proliferation of endothelial cell and breast cancer cells. The number of cells are plotted against TNF-gamma concentration as indicated (TNF-gamma is designated "VEGI" in this figure). Inhibition of the growth of adult bovine aortic endothelial (ABAE) cells (dark circles), but not that of MDA-MB-231 (dark triangles) or MDA-MB-435 (open circles) cells, is shown. The cells were seeded at  $2 \times 10^3$  cells/well in triplicate in 24-well plates. The ABAE cell culture media contained IMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS and (1 ng/ml) FGF-2. The cultures were maintained at 37°C, 5% CO<sub>2</sub>, for 6 days. The cells were then trypsinized, and the number of cells determined by using a Coulter counter. One-fifth of the total number of recovered ABAE cells is shown in order to normalize the comparison with the MDA-MB-231 and MDA-MB-435 cells.

Figure 11 is a photograph of HL 60 cells, with control (Figure 11A) showing the HL60 cells being spread apart; ~~TNF- $\alpha$  (Figure 11B)~~ and TNF-gamma (Figure 11C) induce cell adhesion and cell-cell contact as illustrated by the cells adhering together in the lower right.

Figure 12 illustrates the ability of recombinant TNF-gamma (represented by squares), ~~TNF- $\alpha$  (represented by circles) and TNF- $\beta$~~  and TNF- $\beta$  (represented by triangles) to induce WEHI 164 cell death. Cell death is inversely proportional to the ratio of absorbance at 405 nm to that at 490 nm).

Figure 13 illustrates that TNF-gamma does not significantly bind to two known soluble TNF receptors, namely sTNF RI (p55; solid bars) and sTNF RII (p75; hatched bars).

Figure 14 demonstrates the effect of TNF-gamma on the ability of ABAE cells to form capillary-like tubes on collagen gels. The ability of recombinant TNF-gamma (residues 12-147 as shown in SEQ ID NO:2 and designated "VEGI" in this figure) to inhibit the formation of capillary-like tubes by ABAE cells is shown. The p-values (t-test) given above the columns are obtained by comparing the extent of the capillary-like tube formation by ABAE cells in the presence of various concentrations of TNF-gamma, as indicated, to that when TNF-gamma is absent from the culture media.

Figure 15 shows the inhibition of angiogenesis in collagen gels placed on chicken embryonic chorioallantoic membrane (CAM) by TNF-gamma. The growth of new capillary vessels into collagen gel pellets placed on the CAM was induced by either FGF-2 (100 ng) or VEGF (250 ng). The extent of angiogenesis in the gels was determined by evaluation of the fluorescence intensity of FITC-dextran injected into the CAM circulation. Inhibition of the capillary vessel growth by the recombinant



TNF-gamma (designated "VEGI" in this figure), as indicated by a lower value than 100, is shown. The experiment was carried out in triplicate.

Figure 16 illustrates the inhibition of growth of human breast cancer xenograft tumors in athymic nude mice by TNF-gamma. Mixtures of  
 5 TNF-gamma-overexpressing or vector-transfected CHO cells ( $5 \times 10^6$  cells per injection) and human breast cancer cells ( $1 \times 10^6$  cells per injection) were injected into the mammary fat pads of the nude mice. Tumor sizes (area) were monitored following injection. The sizes of the MDA-MB-231 xenograft tumors ( $\text{mm}^2$ ) were plotted as a function of days post-inoculation (Figure 16A). The sizes of the  
 10 MDA-MB-435 xenograft tumors ( $\text{mm}^2$ ) were plotted as a function of days post-inoculation (Figure 16B). Open circles represent values of tumors co-inoculated with vector-transfected CHO cells, whereas closed circles represent values of tumors co-inoculated with TNF-gamma-transfected CHO cells.

Figure 17 shows an analysis of the TNF-gamma-alpha amino acid sequence  
 15 (SEQ ID NO:2). Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown, as predicted using the default parameters of the recited computer programs. In the "Antigenic Index or Jameson-Wolf" graph, the positive peaks indicate locations of the highly antigenic regions of the TNF-gamma protein,  
 20 i.e., regions from which epitope-bearing peptides of the invention can be obtained.

Figures 18A-D show an alignment of the nucleotide sequences of TNF-gamma-alpha (SEQ ID NO:1) and TNF-gamma-beta (SEQ ID NO:19) constructed by using the computer program BESTFIT set at default parameters.

Figure 19 shows an alignment of the amino acid sequences of TNF-gamma-alpha (SEQ ID NO:2) and TNF-gamma-beta (SEQ ID NO:20) constructed using the  
 25 default parameters of the computer program BESTFIT.

Figures 20A and B illustrate the cDNA (SEQ ID NO:19) and corresponding deduced amino acid sequence (SEQ ID NO:20) of the polypeptide of the TNF-gamma-beta of the present invention. The standard one-letter abbreviations for  
 30 amino acids are used. Amino acids methionine-1 to tryptophan-35 are the predicted intracellular domain. Amino acid residues alanine-36 through alanine-61 (underlined) are the putative transmembrane sequence. Amino acid residues glutamine-62 through leucine-251 (underlined) are the putative transmembrane sequence. Potential asparagine-linked glycosylation sites are marked in Figures 20A and B with a bolded  
 35 asparagine symbol (N) in the TNF-gamma-beta amino acid sequence and a bolded pound sign (#) above the first nucleotide encoding that asparagine residue in the TNF-gamma-alpha nucleotide sequence. Potential N-linked glycosylation sequences

are found at the following locations in the TNF-gamma-beta amino acid sequence: N-133 through N-136 (N-133, Y-134, T-135, N-136) and N-229 through D-232 (N-229, V-230, S-231, D-232). Potential Protein Kinase C (PKC) phosphorylation sites are also marked in Figures 20A and B with a bolded serine or threonine symbol (S or T) in the TNF-gamma-beta amino acid sequence and an asterisk (\*) above the first nucleotide encoding that threonine residue in the TNF-gamma-beta nucleotide sequence. Potential PKC phosphorylation sequences are found at the following locations in the TNF-gamma-beta amino acid sequence: S-23 through R-25 (S-23, C-24, R-25); S-32 through R-34 (S-32, A-33, R-34); T-135 through K-137 (T-135, N-136, K-137); and T-154 through R-156 (T-154, F-155, R-156). Potential Casein Kinase II (CK2) phosphorylation sites are also marked in Figures 20A and B with a bolded serine or threonine symbol (S or T) in the TNF-gamma-beta amino acid sequence and an asterisk (\*) above the first nucleotide encoding the appropriate serine or threonine residue in the TNF-gamma-beta nucleotide sequence. Potential CK2 phosphorylation sequences are found at the following locations in the TNF-gamma-beta amino acid sequence: S-8 through E-11 (S-8, F-9, G-10, E-11); S-187 through E-190 (S-187, Y-188, P-189, E-190); S-200 through E-203 (S-200, V-201, C-202, E-203); S-219 through E-222 (S-219, L-220, Q-221, E-222); S-234 through D-237 (S-234, L-235, V-236, D-237); and T-239 through D-242 (T-239, K-240, E-241, D-242). Potential myristylation sites are also marked in Figures 20A and B with a double underline in the TNF-gamma-beta amino acid sequence. Potential myristylation sequences are found at the following locations in the TNF-gamma-beta amino acid sequence: G-6 through E-11 (G-6, L-7, S-8, F-9, G-10, E-11); G-124 through G-129 (G-124, L-125, A-126, F-127, T-128, K-129); and G-215 through L-220 (G-215, A-216, M-217, F-218, S-219, L-220).

### ***Detailed Description***

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a TNF-gamma-alpha polypeptide having the amino acid sequence shown in Figures 1A and B (SEQ ID NO:2), which was determined by sequencing a cloned cDNA (HUV091). As shown in Figures 2A-2C, the TNF-gamma-alpha polypeptide of the present invention shares sequence homology with human TNF-alpha (SEQ ID NO:3), TNF-beta (SEQ ID NO:4), human lymphotoxin-beta (SEQ ID NO:5) and FAS ligand (SEQ ID NO:6). The TNF-gamma-alpha of the invention functions at least in the inhibition of angiogenesis, as an anti-tumor cytokine-like molecule, as a treatment for arthritis by the inhibition of angiogenesis

and/or endothelial cell proliferation associated with invading pannus in bone and cartilage, as an inducer of NF-kB and c-Jun kinase (JNK), an inducer of cell adhesion, and as an inducer apoptosis (See Examples, particularly Examples 12-15). The nucleotide sequence shown in SEQ ID NO:1 was obtained by sequencing a cDNA clone (HUVE091), which was deposited on October 26, 1994 at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, and given accession number 75927. The deposited plasmid is contained in pBluescript SK(-) plasmid (Stratagene, La Jolla, CA). Further characterization of the protein encoded by clone HUVE091 is presented in copending U. S. Provisional Application Serial No. 60/074,047, filed February 9, 1998; the entire disclosure of which is hereby incorporated by reference.

The present invention also provides isolated nucleic acid molecules comprising a polynucleotide (SEQ ID NO:19) encoding a TNF-gamma-beta polypeptide having the amino acid sequence shown in Figures 20A and B (SEQ ID NO:20), which was determined by sequencing a cloned cDNA (HEMCZ56). The TNF-gamma-beta polypeptide is a splice variant of the TNF-gamma-alpha polypeptide disclosed herein. The nucleotide sequence shown in SEQ ID NO:19 was obtained by sequencing a cDNA clone (HEMCZ56), which was deposited on July 9, 1998 at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, and given accession number 203055. The deposited plasmid is contained in pBluescript SK(-) plasmid (Stratagene, La Jolla, CA).

### ***Nucleic Acid Molecules***

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc., Foster City, CA), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will

cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

5 By "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U), where each thymidine deoxyribonucleotide (T) in the specified deoxyribonucleotide sequence is replaced by the ribonucleotide uridine  
10 (U).

Using the information provided herein, such as the nucleotide sequence in Figures 1A and B (SEQ ID NO:1), or the nucleotide sequence in Figures 20A and B (SEQ ID NO:19) a nucleic acid molecule (i.e., polynucleotide) of the present invention encoding a TNF-gamma-alpha or TNF-gamma-beta polypeptide may be obtained  
15 using standard cloning and screening procedures, such as, for example, those for cloning cDNAs using mRNA as the starting material. For example, polynucleotides encoding TNF-gamma-alpha polypeptides may routinely be obtained from any cell or tissue source that expresses TNF-gamma-alpha, such as, for example, human kidney and umbilical vein endothelial cells. Illustrative of the invention, the nucleic acid molecules described in Figures 1A and B (SEQ ID NO:1) was discovered in a cDNA library derived from human umbilical vein endothelial cells. The cDNA clone  
20 corresponding to TNF-gamma-alpha (clone HUVE091) contains an open reading frame encoding a protein of 174 amino acid residues of which approximately the first 27 amino acids residues are the putative leader sequence such that the mature protein comprises 147 amino acids. The protein exhibits the highest degree of homology at the C-terminus to <sup>Rabbit TNF-alpha</sup> ~~Rabbit TNF-alpha~~ (Ito, H., *et al.*, *DNA* 5:157-165 (1986); GenBank Accession No. M12846; SEQ ID NO:7) with 38% identity and 58% similarity over a  
25 111 amino acid stretch. Sequences conserved throughout the members of the TNF family are also conserved in TNF-gamma (see Figures 2A-2C). The shaded letters indicate conserved amino acid residues. The TNF-gamma mRNA is specifically expressed in human umbilical vein endothelial cells as shown in the RNA blot analysis of Figure 3B.

Further, polynucleotides encoding a TNF-gamma-beta polypeptide may routinely be obtained from induced and resting endothelial cells, umbilical vein,  
35 tonsils, and several other cell and tissue types. Illustrative of the invention, the nucleic acid molecules described in Figures 20A and B (SEQ ID NO:19) was discovered in a cDNA library derived from induced endothelial cells. The cDNA

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clone corresponding to TNF-gamma-<sup>beta</sup> (HEMCZ56) contains an open reading frame encoding a protein of 251 amino acid residues of which approximately the first 35 amino acids residues are the putative intracellular domain and amino acids 36-61 are a putative transmembrane domain and amino acid residues 62-251 are a putative extracellular domain.

The amino acid residues constituting the extracellular, transmembrane, and intracellular domains have been predicted by computer analysis. Thus, as one of ordinary skill would appreciate, the amino acid residues constituting these domains may vary slightly (e.g., by about 1 to about 15 amino acid residues) depending on the criteria used to define each domain.

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figures 1A and B (SEQ ID NO:2), or for the mature polypeptide encoded by the cDNA of the clone designated HUVEO91 deposited as ATCC Deposit No. 75927 on October 26, 1994.

In addition, in accordance with another aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figures 20A and B (SEQ ID NO:20), or for the mature polypeptide encoded by the cDNA of the clone designated HEMCZ56 deposited as ATCC Deposit No. 203055 on July 9, 1998.

By "isolated" nucleic acid molecule(s) or polynucleotide is intended a molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules (polynucleotides) contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules (polynucleotides) include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules (polynucleotides) include *in vivo* or *in vitro* RNA transcripts of the DNA molecules (polynucleotides) of the present invention. Isolated nucleic acid molecules or polynucleotides according to the present invention further include such molecules produced synthetically.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand.

Isolated nucleic acid molecules of the present invention include the polynucleotide sequence depicted in Figures 1A and B (SEQ ID NO:1) encoding the mature TNF-gamma-alpha polypeptide, the polynucleotide sequence depicted in

Figures 20A and B (SEQ ID NO:19) encoding the mature TNF-gamma-beta polypeptide, the polynucleotide sequences contained in deposited clone (HUV091) deposited as ATCC Deposit No. 75927 encoding the mature TNF-gamma-alpha polypeptide, the polynucleotide sequences contained in deposited clone (HEMCZ56) deposited as ATCC Deposit No. 203055 encoding the mature TNF-gamma-beta polypeptide, and polynucleotide sequences which comprise a sequence different from those described above, but which due to the degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figures 1A and B, Figures 20A and B, or the deposited cDNA. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

The amino acid sequence of the complete TNF-gamma-alpha protein includes a leader sequence and a mature protein, as shown in Figures 1A and B (SEQ ID NO:2). More in particular, the present invention provides nucleic acid molecules encoding a mature form of the TNF-gamma-alpha protein. Thus, according to the signal hypothesis, once export of the growing protein chain across the rough endoplasmic reticulum has been initiated, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the complete polypeptide to produce a secreted "mature" form of the protein. Most mammalian cells and even insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species of the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding the mature TNF-gamma-alpha polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 75927. By the "mature TNF-gamma-alpha polypeptide having the amino acid sequence encoded by the cDNA clone in ATCC Deposit No. 75927" is meant the mature form(s) of the TNF-gamma-alpha protein produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the human DNA sequence of the deposited clone.

The polynucleotide which encodes for the mature polypeptide of Figures 20A and B or for the mature polypeptide encoded by the deposited cDNA (HEMCZ56) may include, but is not limited to: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a transmembrane sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments (i.e., portions), analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figures 1A and B, Figures 20A and B, and the polypeptide encoded by the cDNA of the deposited clones. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figures 1A and B, or the mature polypeptide encoded by the cDNA of the deposited clone HUVEO91 as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figures 1A and B, or the polypeptide encoded by the cDNA of the

deposited clone HUVEO91. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

Additionally, the present invention includes polynucleotides encoding the mature polypeptide as shown in Figures 20A and B, or the mature polypeptide encoded by the cDNA of the deposited clone HEMCZ56 as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figures 20A and B, or the polypeptide encoded by the cDNA of the deposited clone HEMCZ56. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figures 1A and 1B or of the coding sequence of the deposited clone HUVEO91. Alternatively, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figures 20A and B or of the coding sequence of the deposited clone HEMCZ56. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNAs (HUVEO91 and HEMCZ56), or the nucleotide sequence shown in Figures 1A and B (SEQ ID NO:1), Figures 20A and B (SEQ ID NO:19), or the complementary strand thereto, is intended fragments at least 15 nt, and more preferably at least 20 nt, still more preferably at least 30 nt, and even more preferably, at least 40, 50, 100, 150, 200, 250, 300, 400, or 500 nt in length. These fragments have numerous uses which include, but are not limited to, diagnostic probes and primers as discussed herein. Of course, larger fragments 50-1500 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNA clone HUVEO91, the deposited cDNA clone HEMCZ56, the nucleotide sequence depicted in Figures 1A and B (SEQ ID NO:1), or the nucleotide sequence depicted in Figures 20 A and B (SEQ ID NO 20). By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of the deposited cDNA clones (HUVEO91 and HEMCZ56), the nucleotide sequence as shown in Figures 1A and B (SEQ ID NO:1), or the nucleotide sequence as shown in Figures 20 A and B.



In specific embodiments, the polynucleotide fragments of the invention encode a polypeptide which demonstrates a functional activity. By a polypeptide demonstrating "functional activity" is meant, a polypeptide capable of displaying one or more known functional activities associated with a complete or mature TNF-gamma polypeptide. Such functional activities include, but are not limited to, biological activity ((e.g., inhibition of angiogenesis, inhibition of endothelial cell proliferation, induction of NF-kB and c-Jun kinase (JNK), induction of cell adhesion, and induction of apoptosis (*See* Examples, particularly Examples 12-15)), antigenicity [ability to bind (or compete with a TNF-gamma polypeptide for binding) to an anti-TNF-gamma antibody], immunogenicity (ability to generate antibody which binds to a TNF-gamma polypeptide), the ability to form polymers with other TNF-gamma polypeptides, and ability to bind to a receptor or ligand for a TNF-gamma polypeptide (e.g. DR3).

The invention also provides nucleic acid molecules having nucleotide sequences related to extensive fragments of SEQ ID NO:1 which have been determined from the following related cDNA clones: HUVEO91 (SEQ ID NO:8), HMPAP05 (SEQ ID NO:9), HSXCA44 (SEQ ID NO:10), HEMFG66 (SEQ ID NO:11), and HELAM93 (SEQ ID NO:12).

The invention also provides nucleic acid molecules having nucleotide sequences related to extensive fragments of SEQ ID NO:19 which have been determined from the following related cDNA clones: HUVEO91P01 (SEQ ID NO:21), HMPTI24R (SEQ ID NO:22), HELAM93R (SEQ ID NO:23), and HEMFG66R (SEQ ID NO:24).

In specific embodiments, the polynucleotide fragments of the invention comprise, or alternatively, consist of, a polynucleotide comprising any portion of at least 30 nucleotides, preferably at least 50 nucleotides, of SEQ ID NO:1 from nucleotide residue 1 to 2442, preferably excluding the nucleotide sequences determined from the abovelisted cDNA clones. Representative examples of the TNF-gamma-alpha polynucleotide fragments of the invention, include fragments that comprise, or alternatively, consist of, nucleotides: 783-1304, 800-1300, 850-1300, 900-1300, 950-1300, 1000-1300, 1050-1300, 1100-1300, 1150-1300, 1200-1300, 1250-1300, 783-1250, 800-1250, 850-1250, 900-1250, 950-1250, 1000-1250, 1050-1250, 1100-1250, 1150-1250, 1200-1250, 783-1200, 800-1200, 850-1200, 900-1200, 950-1200, 1000-1200, 1050-1200, 1100-1200, 1150-1200, 783-1150, 800-1150, 850-1150, 900-1150, 950-1150, 1000-1150, 1050-1150, 1100-1150, 783-1100, 800-1100, 850-1100, 900-1100, 950-1100, 1000-1100, 1050-1100, 783-1050, 800-1050, 850-1050, 900-1050, 950-1050, 1000-1050, 783-1000,

5 In additional specific embodiments, the polynucleotide fragments of the invention comprise, or alternatively, consist of, a polynucleotide comprising any portion of at least 30 nucleotides, preferably at least 50 nucleotides, of SEQ ID NO:19 from nucleotide residue 1 to 1116, preferably excluding the nucleotide sequences determined from the abovelisted cDNA clones (i.e., list from p.25).

15	Representative examples of the TNF-gamma-beta polynucleotide fragments of the invention, include fragments that comprise, or alternatively, consist of, nucleotides 1-1116, 50-1116, 100-1116, 150-1116, 200-1116, 250-1116, 300-1116, 350-1116, 400-1116, 450-1116, 500-1116, 550-1116, 600-1116, 650-1116, 700-1116, 750-1116, 800-1116, 850-1116, 900-1116, 950-1116, 1000-1116,
20	1050-1116, 1-1100, 50-1100, 100-1100, 150-1100, 200-1100, 250-1100, 300-1100, 350-1100, 400-1100, 450-1100, 500-1100, 550-1100, 600-1100, 650-1100, 700-1100, 750-1100, 800-1100, 850-1100, 900-1100, 950-1100, 1000-1100, 1050-1100, 1-1050, 50-1050, 100-1050, 150-1050, 200-1050, 250-1050, 300-1050, 350-1050, 400-1050, 450-1050, 500-1050, 550-1050,
25	600-1050, 650-1050, 700-1050, 750-1050, 800-1050, 850-1050, 900-1050, 950-1050, 1000-1050, 1-1000, 50-1000, 100-1000, 150-1000, 200-1000, 250-1000, 300-1000, 350-1000, 400-1000, 450-1000, 500-1000, 550-1000, 600-1000, 650-1000, 700-1000, 750-1000, 800-1000, 850-1000, 900-1000, 950-1000, 1-950, 50-950, 100-950, 150-950, 200-950, 250-950, 300-950, 350-950,
30	400-950, 450-950, 500-950, 550-950, 600-950, 650-950, 700-950, 750-950, 800-950, 850-950, 900-950, 1-900, 50-900, 100-900, 150-900, 200-900, 250-900, 300-900, 350-900, 400-900, 450-900, 500-900, 550-900, 600-900, 650-900, 700-900, 750-900, 800-900, 850-900, 1-850, 50-850, 100-850, 150-850, 200-850, 250-850, 300-850, 350-850, 400-850, 450-850, 500-850, 550-850, 600-850,
35	650-850, 700-850, 750-850, 800-850, 1-800, 50-800, 100-800, 150-800, 200-800, 250-800, 300-800, 350-800, 400-800, 450-800, 500-800, 550-800, 600-800, 650-800, 700-800, 750-800, 1-750, 50-750, 100-750, 150-750, 200-750, 250-750,

300-750, 350-750, 400-750, 450-750, 500-750, 550-750, 600-750, 650-750, 700-750, 1-700, 50-700, 100-700, 150-700, 200-700, 250-700, 300-700, 350-700, 400-700, 450-700, 500-700, 550-700, 600-700, 650-700, 1-650, 50-650, 100-650, 150-650, 200-650, 250-650, 300-650, 350-650, 400-650, 450-650, 500-650, 550-650, 600-650, 1-600, 50-600, 100-600, 150-600, 200-600, 250-600, 300-600, 350-600, 400-600, 450-600, 500-600, 550-600, 1-550, 50-550, 100-550, 150-550, 200-550, 250-550, 300-550, 350-550, 400-550, 450-550, 500-550, 1-500, 50-500, 100-500, 150-500, 200-500, 250-500, 300-500, 350-500, 400-500, 450-500, 1-450, 50-450, 100-450, 150-450, 200-450, 250-450, 300-450, 350-450, 400-450, 1-400, 50-400, 100-400, 150-400, 200-400, 250-400, 300-400, 350-400, 1-350, 50-350, 100-350, 150-350, 200-350, 250-350, 300-350, 1-300, 50-300, 100-300, 150-300, 200-300, 250-300, 1-250, 50-250, 100-250, 150-250, 200-250, 1-200, 50-200, 100-200, 150-200, 1-150, 50-150, 100-150, 1-100, 50-100, and 1-50 of SEQ ID NO:19 or the complementary polynucleotide strand thereto, or the cDNA contained in the deposited clone HEMCZ56.

Preferred nucleic acid fragments of the present invention also include nucleic acid molecules encoding one or more of the following domains of TNF-gamma-alpha (e.g., as described also in the legend to Figures 1A and 1B): potential asparagine-linked glycosylation sites N-29 through N-32 (N-29, Y-30, T-31, N-32) and N-125 through D-128 (N-125, V-126, S-127, D-128); potential Protein Kinase C (PKC) phosphorylation sites T-32 through K-34 (T-32, N-33, K-34) and T-50 through R-52 (T-50, F-51, R-52); potential Casein Kinase II (CK2) phosphorylation sites S-83 through E-86 (S-83, Y-84, P-85, E-86); S-96 through E-99 (S-96, V-97, C-98, E-99); S-115 through E-118 (S-115, L-116, Q-117, E-118); S-130 through D-133 (S-130, L-131, V-132, D-133); and T-135 through D-138 (T-135, K-136, E-137, D-138); and potential myristylation sites G-20 through K-25 (G-20, L-21, A-22, F-23, T-24, K-25) and G-111 through L-116 (G-111, A-112, M-113, F-114, S-115, L-116) of SEQ ID NO:2.

Among the especially preferred polynucleotides of the invention are those characterized by encoding structural or functional attributes of TNF-gamma. Such polynucleotides encode amino acid residues that comprise alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet forming regions ("beta regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, surface forming regions, and high antigenic index regions (i.e., having an antigenic regions of three or more contiguous amino acid residues each of which having an antigenic index of greater than or equal to 1.5) of

TNF-gamma. Certain preferred regions are those set out in Figure 17, and include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence depicted in Figure 1 (SEQ ID NO:2) using the default parameters of the identified computer programs, such preferred regions include; Garnier-Robson  
 5 alpha-regions, beta-regions, turn-regions, and coil-regions, Chou-Fasman alpha-regions, beta-regions, and coil-regions, Kyte-Doolittle hydrophilic regions and hydrophobic regions, Eisenberg alpha- and beta-amphipathic regions, Karplus-Schulz flexible regions, Emini surface-forming regions and Jameson-Wolf regions of high antigenic index.

10 Data which represent TNF-gamma-beta in a fashion as described above for TNF-gamma-alpha (see Figure 17) may easily be prepared using the amino acid sequence shown in Figures 20A and 20B and in SEQ ID NO:20. As such, each of the abovelisted structural or functional attributes of TNF-gamma listed above (i.e. Garnier-Robson alpha-regions, beta-regions, turn-regions, and coil-regions,  
 15 Chou-Fasman alpha-regions, beta-regions, and coil-regions, Kyte-Doolittle hydrophilic regions and hydrophobic regions, Eisenberg alpha- and beta-amphipathic regions, Karplus-Schulz flexible regions, Emini surface-forming regions and Jameson-Wolf regions of high antigenic index, etc.) apply equally well to TNF-gamma-alpha and TNF-gamma-beta.

20 Certain preferred regions in these regards are set out in Figure 17, but may also be represented or identified by using a tabular representation of the data presented in Figure 17. The DNA\*STAR computer algorithm used to generate Figure 17 (set on the original default parameters) will easily present the data in Figure 17 in such a tabular format. A tabular format of the data in Figure 17 may be used to easily  
 25 determine specific boundaries of a preferred region.

The above-mentioned preferred regions set out in Figure 17 include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence set out in Figures 1A and 1B. As set out in Figure 17, such preferred regions include Garnier-Robson alpha-regions, beta-regions, turn-regions, and  
 30 coil-regions, Chou-Fasman alpha-regions, beta-regions, and coil-regions, Kyte-Doolittle hydrophilic regions and hydrophobic regions, Eisenberg alpha- and beta-amphipathic regions, Karplus-Schulz flexible regions, Emini surface-forming regions and Jameson-Wolf regions of high antigenic index.

35 Among highly preferred fragments in this regard are those that comprise reigons of TNF-gamma-alpha and/or TNF-gamma-beta that combine several structural features, such as several (e.g., 1, 2, 3 or 4) of the features set out above.

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Additional preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding one or more epitope-bearing portions of the TNF-gamma polypeptide. In particular, such nucleic acid fragments of the present invention included nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about Thr-24 to about Asn-32 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Ile-37 to about Ile-45 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Met-54 to about Arg-62 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Gln-63 to about Asp-71 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Glu-57 to about Gly-65 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Val-80 to about Thr-88 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Leu-116 to about Val-124 in SEQ ID NO:2; and a polypeptide comprising amino acid residues from about Asp-133 to about Phe-141 in SEQ ID NO:2. These polypeptide fragments have been determined to bear antigenic epitopes of the TNF-gamma protein by the analysis of the Jameson-Wolf antigenic index, as shown in Figure 17, above. Methods for determining other such epitope-bearing portions of TNF-gamma are described in detail below.

Polypeptide fragments which bear antigenic epitopes of the TNF-gamma-beta protein may be easily determined by one of skill in the art using the above-described analysis of the Jameson-Wolf antigenic index, as shown in Figure 17. Methods for determining other such epitope-bearing portions of TNF-gamma-beta are described in detail below.

Another embodiment of the invention is directed to polynucleotides that hybridize, preferably under stringent hybridization conditions, to a portion of the polynucleotide sequence of a polynucleotide of the invention such as, for instance, the cDNA clone contained in ATCC Deposit No. 75927, the cDNA clone contained in ATCC Deposit 203055 or a TNF-gamma polynucleotide fragment as described herein. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least 15 nucleotides (nt), and more preferably at least 20 nt, still more preferably at least 30 nt, and even more preferably 30-70, or 80-150 nt, or the entire length of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below. Of course, a polynucleotide

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which hybridizes only to a poly A sequence (such as the 3' terminal poly tract of the TNF-gamma cDNA shown in SEQ ID NO:1 or SEQ ID NO:19), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since  
5 such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone generated using oligo dT as a primer).

In preferred embodiments, polynucleotides which hybridize to the reference polynucleotides disclosed herein encode polypeptides which either retain substantially  
10 the same biological function or activity as the mature polypeptide encoded by the polynucleotide sequences depicted in Figures 1A and 1B (SEQ ID NO:1) and/or Figures 20A and B (SEQ ID NO:19), or the cDNAs contained in the deposit.

Alternative embodiments are directed to polynucleotides which hybridize to the reference polynucleotide (i.e., a polynucleotide sequence disclosed herein), but do not  
15 retain biological activity. While these polynucleotides do not retain biological activity, they have uses, such as, for example, as probes for the polynucleotide of SEQ ID NO:1, for recovery of the polynucleotide, as diagnostic probes, and as PCR primers.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the  
20 TNF-gamma protein. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism (*Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985)). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or  
25 additions of the polynucleotide sequences described herein (including fragments). The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid  
30 substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the TNF-gamma protein or portions thereof. Also especially preferred in this regard are conservative substitutions.

Further embodiments of the invention are directed to isolated nucleic acid  
35 molecules comprising a polynucleotide sequence at least 70% or at least 80% identical, more preferably at least 90% identical, and still more preferably at least 95%, 96%, 97%, 98% or 99% identical to a polynucleotide having a nucleotide sequence selected

Further embodiments of the invention are directed to isolated nucleic acid molecules comprising a polynucleotide sequence at least 70% or at least 80% identical, more preferably at least 90% identical, and still more preferably at least 95%, 96%, 97%, 98% or 99% identical to a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding the TNF-gamma-beta polypeptide having the complete amino acid sequence in SEQ ID NO:20 (i.e., positions 1 to 251 of SEQ ID NO:20); (b) a nucleotide sequence encoding the TNF-gamma-beta polypeptide having the complete amino acid sequence in SEQ ID NO:20 excepting the N-terminal methionine (i.e., positions 2 to 251 of





entire nucleotide sequence shown in Figures 1A and B (SEQ ID NO:1) and Figures 20 A and B (SEQ ID NO:19), or any fragment as described herein.

As a practical matter, whether any particular nucleic acid molecule is at least 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the  
5 nucleotide sequence shown in Figures 1A and B (SEQ ID NO:1), Figures 20A and B (SEQ ID NO:19), or to the nucleotide sequence of the deposited cDNA clones can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics  
10 Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a  
15 reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also referred to as a global  
20 sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty  
25 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for 5' and 3' truncations of the subject sequence  
30 when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. A determination of whether a nucleotide is matched/aligned is determined by results of  
35 the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the

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purposes of this embodiment. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score. For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

In further embodiments, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

In further embodiments, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:20 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The present application is directed to nucleic acid molecules at least 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% identical to the polynucleotide sequence shown in Figures 1A and B (SEQ ID NO:1), Figures 20A and B (SEQ ID NO:19), or to the nucleic acid sequence of the deposited cDNA clones, or fragments thereof, irrespective of whether they encode a polypeptide having TNF-gamma functional activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having TNF-gamma functional activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid

molecules of the present invention that do not encode a polypeptide having TNF-gamma functional activity include, *inter alia*, (1) isolating the TNF-gamma gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the TNF-gamma gene, as described in Verma *et al.*, *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, N.Y. (1988); and (3) Northern Blot analysis for detecting TNF-gamma mRNA expression in specific tissues.

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make phenotypically silent amino acid substitutions is provided in J.U. Bowie et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

5 Additional embodiments of the invention are directed to isolated nucleic acid molecules comprising a polynucleotide which encodes the amino acid sequence of a TNF-gamma polypeptide (e.g., a TNF-gamma polypeptide fragment described herein) having an amino acid sequence which contains at least one conservative amino acid substitution, but not more than 50 conservative amino acid substitutions, even more  
10 preferably, not more than 40 conservative amino acid substitutions, still more preferably, not more than 30 conservative amino acid substitutions, and still even more preferably, not more than 20 conservative amino acid substitutions, 10-20 conservative amino acid substitutions, 5-10 conservative amino acid substitutions, 1-5 conservative amino acid substitutions, 3-5 conservative amino acid substitutions, or 1-  
15 3 conservative amino acid substitutions. Of course, in order of ever-increasing preference, it is highly preferable for a polynucleotide which encodes the amino acid sequence of a TNF-gamma polypeptide to have an amino acid sequence which contains not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 conservative amino acid substitutions.

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### ***Polynucleotide Assays***

The invention also encompasses the use of TNF-gamma polynucleotides to detect complementary polynucleotides, such as, for example, as a diagnostic reagent for detecting diseases or susceptibility to diseases related to the presence of mutated  
25 TNF-gamma-alpha or TNF-gamma-beta. Such diseases are related to an under-expression of TNF-gamma-alpha or TNF-gamma-beta, such as, for example, abnormal cellular proliferation such as tumors and cancers.

Individuals carrying mutations in the human TNF-gamma gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and  
30 autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, *Nature*, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding TNF-gamma-alpha or TNF-gamma-beta can be used to identify and analyze TNF-gamma  
35 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can

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Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, Science, 230:1242 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. § 112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention also relates to vectors which include the isolated polynucleotides of the present invention, host cells which are genetically engineered with the recombinant vectors, or which are otherwise engineered to produce the

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the TNF-gamma genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operably associated with an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli lac* or *trp*, the phage lambda P promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila* S2 and Sf9; animal cells such as CHO, COS or Bowes melanoma, adenoviruses, plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably associated with the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pHE4-5 (ATCC Accession No. 209311; and variations thereof), pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pBluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include *lacI*, *lacZ*, T3, T7, gpt, lambda P, P, and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retroviruses, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

In addition to encompassing host cells containing the vector constructs discussed herein, the invention also encompasses primary, secondary, and immortalized host cells of vertebrate origin, particularly mammalian origin, that have

been engineered to delete or replace endogenous genetic material (e.g., TNF-gamma coding sequence), and/or to include genetic material (e.g., heterologous polynucleotide sequences) that is operably associated with TNF-gamma polynucleotides of the invention, and which activates, alters, and/or amplifies endogenous TNF-gamma polynucleotides. For example, techniques known in the art may be used to operably associate heterologous control regions (e.g., promoter and/or enhancer) and endogenous TNF-gamma polynucleotide sequences via homologous recombination (see, e.g., U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra et al., Nature 342:435-438 (1989), the disclosures of each of which are incorporated by reference in their entireties).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), <sup>alpha-factor</sup> ~~alpha-factor~~, acid phosphatase, or heat shock proteins,



among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, for example, stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative, but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman (*Cell* **23**:175 (1981)), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites,

transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The TNF-gamma polypeptides can be recovered and purified from  
 5 recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature  
 10 protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant,  
 15 insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The invention encompasses TNF-gamma-alpha and TNF-gamma-beta polypeptides  
 20 which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen  
 25 bromide, trypsin, chymotrypsin, papain, V8 protease,  $\text{NaBH}_4$ ; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, polypeptides of the invention can be chemically synthesized using techniques known in the art. For example, a peptide corresponding to a fragment of  
 30 the TNF-gamma-alpha and TNF-gamma-beta polypeptides of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the lats sequence. Non-classical amino acids include but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx,  
 35 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline,

At least fifteen TNF-gamma-alpha expression constructs have been generated by the inventors herein to facilitate the production of TNF-gamma polypeptides of several sizes and in several systems. Of these, four have been constructed which encode a full-length TNF-gamma polypeptide. The full-length constructs are: (i) pQE9TNFg-27/147, (ii) pQE70TNFg, (iii) pC1TNFg, and pcDNA3TNFg. In the case of the first expression construct listed (pQE9TNFg-27/147), the construct was used to produce a full-length TNF-gamma-alpha polypeptide with an N-terminal six histidine amino acid tag according to the method of Example 1. A full-length TNF-gamma-alpha polypeptide lacking the histidine tag was produced in bacteria by using the pQE70TNFg construct essentially as was done in Example 1. In addition, a full-length TNF-gamma-alpha polypeptide lacking a histidine tag was produced in mammalian cells by using either the pC1TNFg or pcDNA3TNFg constructs according to the method of Example 3. Further, the mature TNF-gamma-alpha polypeptide was produced and secreted from mammalian cells under direction of the interleukin (IL)-6 signal peptide from a construct designated pcDNA3/IL6TNFg-1/149 (see Example 11).

Sub c5 The remaining TNF-gamma-alpha expression constructs were used to express various TNF-gamma mutants from bacterial, baculoviral, and mammalian systems. Four N-terminal deletion mutations have been generated using the pQE60 bacterial expression vector. These N-terminal deletion mutation constructs are: (i) pQE60TNFg-3/147 (representing a possible mature TNF-gamma polypeptide; the polypeptide expressed by this construct is identical to amino acid residues 107-251 of the TNF-gamma-beta of SEQ ID NO:20), (ii) pQE60TNFg12/147 (representing amino acid residues 12-147 of SEQ ID NO:2 and residues 116-251 of SEQ ID NO:20), (iii) pQE60TNFg22/147 (representing amino acid residues 22-147 and residues 126-251 of SEQ ID NO:20), and (iv) pQE60TNFg28/147 (representing amino acid residues 28-147 and residues 132-251 of SEQ ID NO:20). Each of these expression constructs can be used to produce a TNF-gamma polypeptide in a bacterial system which exhibits an N-terminal deletion of 25, 39, 49, and 55 amino acids, respectively, with regard to the full-length TNF-gamma-alpha polypeptide or an N-terminal deletion of 106, 115, 125, and 131 amino acids, respectively, with regard to the full-length TNF-gamma-beta polypeptide.

Further N-terminal deletion mutation bacterial expression constructs have been generated. A construct designated pHE4 VEGI T30-L174 has been generated using the bacterial expression vector pHE4 to express amino acids threonine-30 to leucine-174 of the TNF-gamma-alpha sequence shown in Figures 1A and 1B (residues threonine-3 to leucine-147 of SEQ ID NO:2) which correspond exactly to amino acid residues threonine-107 to leucine-251 of the TNF-gamma-beta sequence shown in Figures 20A and 20B (residues threonine-107 to leucine-251 of SEQ ID NO:20). Additional bacterial expression constructs generated include pQE9.VEGI.his.T28-L174, pHE4.VEGI.T28-L174, pHE4.VEGI.T51-L174, and pHE4.VEGI.T58-L174. These constructs are based on either the pQE9 or pHE4 bacterial expression vectors. The construct designations indicate the expression vector, the gene name, and the amino acid residues expressed by the construct (e.g. pQE9.VEGI.T28-L174 indicates that the pQE9 bacterial expression vector is used to express amino acids threonine (T)-28 through leucine (L)-174 of the TNF-gamma-alpha polypeptide (VEGI is a laboratory designation for TNF-gamma-alpha)).

A TNF-gamma expression construct has been generated which can be used to produce a secreted mature TNF-gamma polypeptide from a mammalian system. The construct has been designated pC1/IL6TNFg-3/147. It encodes the signal peptide from the human IL-6 gene fused to the mature TNF-gamma sequence. A similar construct has been generated which contains the <sup>CK-beta 8</sup> ~~CK-beta~~ signal peptide (amino acids -21 to -1 of the <sup>CK-beta 8</sup> ~~CK-beta~~ sequence disclosed in published PCT application PCT/US95/09058; filed 6/23/95) fused to the amino terminus of amino acids 12-149 of TNF-gamma-alpha (SEQ ID NO:2; that is, amino acids 116-251 of TNF-gamma-beta (SEQ ID NO:20)) in the context of the pC4 mammalian expression vector. This construct has been designated <sup>pC4/CK-beta 8 TNFg 12/147</sup> ~~pC4/CK-beta 8 TNFg 12/147~~. A variant of this construct has been generated which can be used to express amino acids 12-147 of TNF-gamma fused to the human IgG Fc region at the TNF-gamma carboxy terminus. This fusion protein is also secreted under the direction of the <sup>CK-beta 8</sup> ~~CK-beta~~ signal peptide and has been designated <sup>pC4/CK-beta 8 TNFg 12/147/Fc</sup> ~~pC4/CK-beta 8 TNFg 12/147/Fc~~. The sequence of the human Fc portion of the fusion molecule is shown in SEQ ID NO:18. Other sequences could be used which are known to those of skill in the art.

Amino acids -3 to 147 of TNF-gamma-alpha (SEQ ID NO:2; which correspond to amino acid residues 102 to 251 of TNF-gamma-beta (SEQ ID NO:20)) can be expressed and secreted from a baculovirus system by using a construct designated pA2GPTNFg-3/147. This expression construct encodes the mature TNF-gamma coding sequence fused at its amino terminus to the baculoviral GP signal peptide.

Two retroviral TNF-gamma expression constructs have also been generated. The first of these has been designated pG1SamEN/TNFg-3/149. This expression construct can be used to produce full-length TNF-gamma protein from a mammalian system. A related construct, <sup>pg1SamENCK-beta8TNFg 12/149</sup> ~~pg1SamENCK-beta8TNFg-12/149~~, has been generated which can be used to produce and secrete mature TNF-gamma protein from a mammalian system under the direction of the <sup>CK-beta 8</sup> ~~CK-beta 8~~ signal peptide.

Further polypeptides of the present invention include polypeptides which have at least 90% similarity, more preferably at least 95% similarity, and still more preferably at least 96%, 97%, 98% or 99% similarity to those described above. The polypeptides of the invention also comprise those which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide encoded by the deposited cDNA or to the polypeptide of SEQ ID NO:2, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

### ***Polypeptides and Fragments***

The present invention further relates to an isolated TNF-gamma-alpha polypeptide which has the deduced amino acid sequence of Figures 1A and 1B (SEQ ID NO:2) or which has the amino acid sequence encoded by the deposited cDNA HUVEO91, as well as fragments, analogs and derivatives of such polypeptide.

The present invention also relates to a TNF-gamma-beta polypeptide which has the deduced amino acid sequence of Figures 20A and 20B (SEQ ID NO:20) or which has the amino acid sequence encoded by the deposited cDNA HEMCZ56, as well as fragments, analogs and derivatives of such polypeptide.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to a point within the range of near complete (e.g., >90% pure) to complete (e.g., >99% pure) homogeneity. The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Also intended as an "isolated polypeptide" are polypeptides that have been purified partially or substantially from a recombinant host cell. For example, a recombinantly produced version of a TNF-gamma polypeptide can be substantially purified by the one-step method described by Smith and Johnson (*Gene* 67:31-40 (1988)). Such polynucleotides could be part of a vector

and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. Isolated polypeptides and polynucleotides according to the present invention also include such molecules produced naturally or synthetically. Polypeptides and polynucleotides of the invention also can be purified from natural or recombinant sources using anti-TNF-gamma antibodies of the invention in methods which are well known in the art of protein purification.

*Subcl* The terms "fragment," "derivative" and "analog" when referring to the polypeptides of Figures 1A and 1B or Figures 20A and 20B, and those polypeptides encoded by the deposited cDNAs, means a polypeptide which retains a TNF-gamma functional activity, i.e., displays one or more functional activities associated with a full-length and/or mature TNF-gamma polypeptide disclosed in Figures 1A and B (SEQ ID NO:2), Figures 20 A and B (SEQ ID NO:20), and/or enclosed by one or both of the deposited clones (HUVEO91 and HEMCZ56). As one example, such fragments, derivatives, or analogs, which have the desired immunogenicity or antigenicity can be used, for example, in immunoassays, for immunization, for inhibition of TNF-gamma activity, etc. Thus, a specific embodiment of the invention relates to a TNF-gamma fragment that can be bound by an antibody that specifically binds the TNF-gamma polypeptide sequence disclosed in Figures 1A and B (SEQ ID NO:2), Figures 20 A and B (SEQ ID NO:20) ), and/or which is enclosed by one or both of the deposited clones (HUVEO91 and HEMCZ56).

As another example, TNF-gamma fragments, derivatives or analogs which have TNF-gamma biological activity (e.g., a mature TNF-gamma-alpha polypeptide or the extracellular domain of a TNF-gamma-beta polypeptide) are provided. TNF-gamma fragments, derivatives, and analogs that retain, or alternatively lack a desired TNF-gamma property of interest (e.g., inhibition of cell proliferation, tumor inhibition, inhibition of angiogenesis, anti-arthritis by the inhibition of angiogenesis and/or endothelial cell proliferation associated with invading pannus in bone and cartilage, an inducer of NF-kB and c-Jun kinase (JNK), an inducer of cell adhesion, and as an inducer apoptosis (*See Examples, particularly Examples 12-15*)) can be used as inducers or inhibitors, respectively, of such properties and its physiological correlates.

The polypeptides of the invention may exist as a membrane bound receptor having a transmembrane region and an intra- and extracellular region or they may exist in soluble form wherein the transmembrane domain is lacking. One example of such a form of TNF-gamma is the TNF-gamma-beta polypeptide sequence shown in Figures

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20 A and B (SEQ ID NO:20) which contains a transmembrane, intracellular and extracellular domain.

It will be recognized in the art that some amino acid sequences of the TNF-gamma polypeptide can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Thus, the invention further includes variations of the TNF-gamma polypeptide which show substantial TNF-gamma polypeptide activity or which include regions of TNF-gamma protein such as the polypeptide fragments disclosed herein. Such variants include deletions, insertions, inversions, repeats, and type substitutions selected according to general rules known in the art so as have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change (Bowie *et al.*, *Science* **247**:1306-1310 (1990)). The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described by Bowie and coworkers (*supra*) and the references cited therein. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Thus, the fragment, derivative or analog of the polypeptide of SEQ ID NO:2, or of SEQ ID NO:20, or those encoded by the deposited cDNAs, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature form of the TNF-gamma polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide

(for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the above form of the polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the above form of the polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Thus, the TNF-gamma of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation. As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine



Embodiments of the invention are directed to polypeptides which comprise the amino acid sequence of a TNF-gamma polypeptide <sup>described herein</sup> ~~described herein~~, but having an amino acid sequence which contains at least one conservative amino acid substitution, but not more than 50 conservative amino acid substitutions, even more preferably, not more than 40 conservative amino acid substitutions, still more preferably, not more than 30 conservative amino acid substitutions, and still even more preferably, not more than 20 conservative amino acid substitutions, when compared with the TNF-gamma polynucleotide sequence described herein. Of course, in order of ever-increasing preference, it is highly preferable for a peptide or polypeptide to have an amino acid sequence which comprises the amino acid sequence of a TNF-gamma polypeptide, which contains at least one, but not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 conservative amino acid substitutions.

In further specific embodiments, the number of substitutions, additions or deletions in the amino acid sequence of Figures 1A and B (SEQ ID NO:2), Figures 20 A and B (SEQ ID NO:20), a polypeptide sequence encoded by the deposited clones, and/or any of the polypeptide fragments described herein (e.g., the extracellular domain or intracellular domain) is 75, 70, 60, 50, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 or 150-50, 100-50, 50-20, 30-20, 20-15, 20-10, 15-10, 10-1, 5-10, 1-5, 1-3 or 1-2.

To improve or alter the characteristics of TNF-gamma polypeptides, protein engineering may be employed. Recombinant DNA technology known to those skilled in the art can be used to create novel mutant proteins or muteins including single or multiple amino acid substitutions, deletions, additions or fusion proteins. Such modified polypeptides can show, e.g., enhanced activity or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions.

Thus, the invention also encompasses TNF-gamma derivatives and analogs that have one or more amino acid residues deleted, added, or substituted to generate TNF-gamma polypeptides that are better suited for expression, scale up, etc., in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges; N-linked glycosylation sites can be altered or eliminated to achieve, for example, expression of a homogeneous product that is more easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety of amino acid substitutions at one or both of the first or third amino acid positions on any one or more of the glycosylation recognition sequences in the TNF-gamma polypeptides of

the invention, and/or an amino acid deletion at the second position of any one or more such recognition sequences will prevent glycosylation of the TNF-gamma polypeptide at the modified tripeptide sequence (see, e.g., Miyajimo et al., EMBO J 5(6):1193-1197).

5 Amino acids in the TNF-gamma protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then  
10 tested for biological activity such as receptor binding or *in vitro* proliferative activity.

Of special interest are substitutions of charged amino acids with other charged or neutral amino acids which may produce proteins with highly desirable improved characteristics, such as less aggregation. Aggregation may not only reduce activity but also be problematic when preparing pharmaceutical formulations, because  
15 aggregates can be immunogenic (Pinckard, et al., *Clin. Exp. Immunol.* 2:331-340 (1967); Robbins, et al., *Diabetes* 36:838-845 (1987); Cleland, et al., *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993)).

Since TNF-gamma is a member of the TNF-related protein family, to modulate rather than completely eliminate biological activities of TNF-gamma preferably  
20 additions, substitutions, or deletions are made in sequences encoding amino acids in the conserved TNF-like domain, i.e., in positions 17-147 of SEQ ID NO:2 or positions 121-251 of SEQ ID NO:20, more preferably in residues within this region which are not conserved in all members of the TNF-related protein family (see Figures 2A-2C). Also forming part of the present invention are isolated polynucleotides  
25 comprising nucleic acid sequences which encode the above TNF-gamma variants.

Several amino acids of the TNF-gamma polypeptide are highly conserved across the known members of the TNF-related protein family. By making a specific mutation in TNF-gamma in such residues as tyrosine-15 (as numbered in SEQ IN NO:2), leucine-35, glycine-41, tyrosine-43, tyrosine-46, glutamine-48, leucine-90,  
30 leucine-116, glycine-119, aspartic acid-120, phenylalanine-141, phenylalanine-142, and leucine-147, it is likely that an noticeable effect on biological activity will be observed. These identical amino acid residues are, of course, present in the corresponding positions of TNF-gamma-beta shown in SEQ ID NO:20.

The present invention also encompasses fragments of the above-described  
35 TNF-gamma polypeptides. Polypeptide fragments of the present invention include polypeptides comprising an amino acid sequence contained in SEQ ID NO:2, encoded by the cDNA contained in the deposited clone (HUVEO91), or encoded by nucleic

acids which hybridize (e.g. under stringent hybridization conditions) to the nucleotide sequence contained in the deposited clones, that shown in Figures 1A and 1B (SEQ ID NO:1) and/or Figures 20A and 20B (SEQ ID NO:19), or the complementary strand thereto.

5 Polypeptide fragments may be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, included, for example, fragments that comprise or alternatively, consist of, from about amino acid residues, 1 to 20, 21 to 40, 41 to 60, 61 to 83, 84 to 100, 101  
10 to 120, 121 to 140, 141 to 160, 160 to 167, 161 to 174, 161 to 180, 181 to 200, 201 to 220, 221 to 240, 241 to 251 of SEQ ID NO:2 and/or SEQ ID NO:20. Moreover, polypeptide fragments can be at least about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 or 150 amino acids in length. In this context "about" includes the particularly recited ranges, larger or smaller by several (i.e. 5, 4, 3, 2 or 1) amino  
15 acids, at either extreme or at both extremes.

In other embodiments, the fragments or polypeptides of the invention (i.e., those described herein) are not larger than 250, 225, 200, 185, 175, 170, 165, 160, 155, 150, 145, 140, 135, 130, 125, 120, 115, 110, 105, 100, 90, 80, 75, 60, 50, 40, 30 or 25 amino acids residues in length.

20 Further preferred embodiments encompass polypeptide fragments comprising, or alternatively consisting of, the mature domain of TNF-gamma-alpha (amino acid residues 1-147 of SEQ ID NO:2), the intracellular domain of TNF-gamma-beta (amino acid residues 1-35 of SEQ ID NO:20), the transmembrane domain of TNF-gamma-beta (amino acid residues 36-61 of SEQ ID NO:20), and/or the extracellular  
25 domain of TNF-gamma-beta (amino acid residues 62-251 of SEQ ID NO:20).

In specific embodiments, polypeptide fragments of the invention comprise, or alternatively, consist of, amino acid residues leucine-35 to valine-49, tryptophan-104 to leucine-116, glycine-119 to serine-127, lysine-139 to leucine-147 of SEQ ID NO:2). These domains are regions of high identity identified by comparison of the  
30 TNF family member polypeptides shown in Figures 2A, 2B, and 2C.

Among the especially preferred fragments of the invention are fragments characterized by structural or functional attributes of TNF-gamma. Such fragments include amino acid residues that comprise alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet-forming regions ("beta-regions"), turn  
35 and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, surface forming regions, and high antigenic index regions (i.e.,

regions of polypeptides consisting of amino acid residues having an antigenic index of or equal to greater than 1.5, as identified using the default parameters of the Jameson-Wolf program) of TNF-gamma. Certain preferred regions are those disclosed in Figure 17 and include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence depicted in Figures 1A and B, such preferred regions include; Garnier-Robson predicted alpha-regions, beta-regions, turn-regions, and coil-regions; Chou-Fasman predicted alpha-regions, beta-regions, turn-regions, and coil-regions; Kyte-Doolittle predicted hydrophilic and hydrophobic regions; Eisenberg alpha and beta amphipathic regions; Emini surface-forming regions; and Jameson-Wolf high antigenic index regions, as predicted using the default parameters of these computer programs. Polynucleotides encoding these polypeptides are also encompassed by the invention.

Additionally, analogs of the invention include a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

In another embodiment, the invention provides a TNF-gamma polypeptide (e.g., fragment) comprising, or alternatively, consisting of, an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope". The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes (see, for instance, Geysen, *et al.*, *Proc. Natl. Acad. Sci. USA* **81**:3998-4002 (1983)).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein (see, for instance, Sutcliffe, J. G., *et al.*, *Science* **219**:660-666 (1983)). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl termini. Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a

polypeptide of the invention (see, for instance, Wilson, *et al*, *Cell* 37:767-778 (1984)).

Sub c1 Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. Non-limiting examples of antigenic polypeptides or peptides that can be used to generate TNF-gamma-specific antibodies include: a polypeptide comprising amino acid residues from about Thr-24 to about Asn-32 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Ile-37 to about Ile-45 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Met-54 to about Arg-62 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Gln-63 to about Asp-71 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Glu-57 to about Gly-65 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Val-80 to about Thr-88 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about Leu-116 to about Val-124 in SEQ ID NO:2; and a polypeptide comprising amino acid residues from about Asp-133 to about Phe-141 in SEQ ID NO:2. These polypeptide fragments have been determined to bear antigenic epitopes of the TNF-gamma protein by the analysis of the Jameson-Wolf antigenic index, as shown in Figure 17, above.

One of ordinary skill in the art may easily determine antigenic regions for TNF-gamma-beta by using data prepared through a DNA\*STAR analysis of the TNF-gamma-beta polypeptide sequence (SEQ ID NO:20) using the default parameters and selecting regions with a high antigenic index as described above.

In another aspect, the invention provides peptides and polypeptides comprising epitope-bearing portions of the polypeptides of the present invention. These epitopes are immunogenic or antigenic epitopes of the polypeptides of the present invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response *in vivo* when the whole polypeptide of the present invention, or fragment thereof, is the immunogen. On the other hand, a region of a polypeptide to which an antibody can bind is defined as an "antigenic determinant" or "antigenic epitope." The number of *in vivo* immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, e.g., Geysen, et al. (1983) Proc. Natl. Acad. Sci. USA 81:3998-4002. However, antibodies can be made to any antigenic epitope, regardless of whether it is an immunogenic epitope, by using methods such as phage display. See e.g., Petersen G. et al. (1995) Mol. Gen. Genet. 249:425-431. Therefore, included in the present invention are both immunogenic epitopes and antigenic epitopes.

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A list of exemplified amino acid sequences comprising immunogenic epitopes is described above. It is pointed out that the list of immunogenic epitopes only lists amino acid residues comprising epitopes predicted to have the highest degree of antigenicity using the algorithm of Jameson and Wolf, (1988) *Comp. Appl. Biosci.* 4:181-186 (said references incorporated by reference in their entireties). The Jameson-Wolf antigenic analysis was performed using the computer program PROTEAN, using default parameters (Version 3.11 for the Power MacIntosh, DNASTAR, Inc., 1228 South Park Street Madison, WI). Portions of polypeptides not listed in the above list of immunogenic epitopes are not considered non-immunogenic. The immunogenic epitopes listed above is an exemplified list, not an exhaustive list, because other immunogenic epitopes are merely not recognized as such by the particular algorithm used. Amino acid residues comprising other immunogenic epitopes may be routinely determined using algorithms similar to the Jameson-Wolf analysis or by *in vivo* testing for an antigenic response using methods known in the art. *See, e.g.,* Geysen et al., *supra*; U.S. Patents 4,708,781; 5,194,392; 4,433,092; and 5,480,971 (said references incorporated by reference in their entireties).

It is particularly pointed out that the amino acid sequences listed above comprise immunogenic epitopes. The list of immunogenic epitopes lists only the critical residues of immunogenic epitopes determined by the Jameson-Wolf analysis. Thus, additional flanking residues on either the N-terminal, C-terminal, or both N- and C-terminal ends may be added to the sequences listed above to generate an epitope-bearing polypeptide of the present invention. Therefore, the immunogenic epitopes listed above may include additional N-terminal or C-terminal amino acid residues. The additional flanking amino acid residues may be contiguous flanking N-terminal and/or C-terminal sequences from the polypeptides of the present invention, heterologous polypeptide sequences, or may include both contiguous flanking sequences from the polypeptides of the present invention and heterologous polypeptide sequences.

Polypeptides of the present invention comprising immunogenic or antigenic epitopes are at least 7 amino acids residues in length. "At least" means that a polypeptide of the present invention comprising an immunogenic or antigenic epitope may be 7 amino acid residues in length or any integer between 7 amino acids and the number of amino acid residues of the full length polypeptides of the invention. Preferred polypeptides comprising immunogenic or antigenic epitopes are at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acid residues in length. However, it is pointed out that each and every integer between 7

and the number of amino acid residues of the full length polypeptide are included in the present invention.

The immuno and antigenic epitope-bearing fragments may be specified by either the number of contiguous amino acid residues, as described above, or further specified by N-terminal and C-terminal positions of these fragments on the amino acid sequence of SEQ ID NO:2. Every combination of a N-terminal and C-terminal position that a fragment of, for example, at least 7 or at least 15 contiguous amino acid residues in length could occupy on the amino acid sequence of SEQ ID NO:2 is included in the invention. Again, "at least 7 contiguous amino acid residues in length" means 7 amino acid residues in length or any integer between 7 amino acids and the number of amino acid residues of the full length polypeptide of the present invention. Specifically, each and every integer between 7 and the number of amino acid residues of the full length polypeptide are included in the present invention. Further, immuno- and antigenic epitope-bearing fragments may be specified in the same way for TNF-gamma-beta by using the techniques described herein.

Immunogenic and antigenic epitope-bearing polypeptides of the invention are useful, for example, to make antibodies which specifically bind the polypeptides of the invention, and in immunoassays to detect the polypeptides of the present invention. The antibodies are useful, for example, in affinity purification of the polypeptides of the present invention. The antibodies may also routinely be used in a variety of qualitative or quantitative immunoassays, specifically for the polypeptides of the present invention using methods known in the art. *See, e.g.,* Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press; 2nd Ed. 1988).

The epitope-bearing polypeptides of the present invention may be produced by any conventional means for making polypeptides including synthetic and recombinant methods known in the art. For instance, epitope-bearing peptides may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a simple method for the synthesis of large numbers of peptides, such as 10-20 mgs of 248 individual and distinct 13 residue peptides representing single amino acid variants of a segment of the HA1 polypeptide, all of which were prepared and characterized (by ELISA-type binding studies) in less than four weeks (Houghten, R. A. *Proc. Natl. Acad. Sci. USA* **82**:5131-5135 (1985)). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten and coworkers (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in





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5           Fragments (i.e., portions) of the TNF-gamma polypeptides of the present on  
have uses which include, but are not limited to, intermediates for producing full-length  
polypeptides.

In the present case, since the proteins of the invention are members of the TNF polypeptide family, deletions of N-terminal amino acids up to the leucine residue at position 35 of SEQ ID NO:2 (which corresponds exactly to the leucine residue at position 134 of SEQ ID NO:20) may retain some biological activity such as regulation of growth and differentiation of many types of hematopoietic and endothelial cells. Polypeptides having further N-terminal deletions including the leucine-36 residue in SEQ ID NO:2 (corresponding to leucine-135 in SEQ ID NO:20) would not be expected to retain such biological activities because it is known that this residue in TNF-related polypeptides is in the beginning of the conserved domain required for biological activities.

However, even if deletion of one or more amino acids from the N-terminus of a full-length TNF-gamma polypeptide results in modification or loss of one or more biological functions of the polypeptide, other biological activities may still be retained. Thus, the ability of the shortened polypeptide to induce and/or bind to antibodies which recognize the full-length or mature form of the polypeptide generally will be

retained when less than the majority of the residues of the full-length or mature polypeptide are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the amino terminus of the amino acid sequence of the TNF-gamma-alpha shown in SEQ ID NO:2, up to the leucine residue at position number 35, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising the amino acid sequence of residues  $n^1$ -149 of SEQ ID NO:2, where  $n^1$  is an integer in the range of -27 to 35, and 35 is the position of the first residue from the N-terminus of the complete TNF-gamma polypeptide (shown in SEQ ID NO:2) believed to be required for regulation of growth and differentiation of many types of hematopoietic and endothelial cells.

In specific embodiments, the invention provides polynucleotides encoding polypeptides comprising, or alternatively, consisting of, the amino acid sequence of residues: -27 to 147, -26 to 147, -25 to 147, -24 to 147, -23 to 147, -22 to 147, -21 to 147, -20 to 147, -19 to 147, -18 to 147, -17 to 147, -16 to 147, -15 to 147, -14 to 147, -13 to 147, -12 to 147, -11 to 147, -10 to 147, -9 to 147, -8 to 147, -7 to 147, -6 to 147, -5 to 147, -4 to 147, -3 to 147, -2 to 147, -1 to 147, 1 to 147, 2 to 147, 3 to 147, 4 to 147, 5 to 147, 6 to 147, 7 to 147, 8 to 147, 9 to 147, 10 to 147, 11 to 147, 12 to 147, 13 to 147, 14 to 147, 15 to 147, 16 to 147, 17 to 147, 18 to 147, 19 to 147, 20 to 147, 21 to 147, 22 to 147, 23 to 147, 24 to 147, 27 to 147, 26 to 147, 27 to 147, 28 to 147, 29 to 147, 30 to 147, 31 to 147, 32 to 147, 33 to 147, 34 to 147, and 35 to 147 of SEQ ID NO:2. Polynucleotides encoding these polypeptides are also encompassed by the invention.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the amino terminus of the amino acid sequence of the TNF-gamma-beta shown in SEQ ID NO:20, up to the leucine residue at position number 134, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising the amino acid sequence of residues  $n^2$ -251 of SEQ ID NO:20, where  $n^2$  is an integer in the range of 1 to 134, and 135 is the position of the first residue from the N-terminus of the complete TNF-gamma-beta polypeptide (shown in SEQ ID NO:20) believed to be required for regulation of growth and differentiation of many types of hematopoietic and endothelial cells activity of the TNF-gamma-beta polypeptide.

In specific embodiments, the invention provides polynucleotides encoding polypeptides comprising, or alternatively, consisting of, the amino acid sequence of residues: 1 to 251, 2 to 251, 3 to 251, 4 to 251, 5 to 251, 6 to 251, 7 to 251, 8 to 251, 9 to 251, 10 to 251, 11 to 251, 12 to 251, 13 to 251, 14 to 251, 15 to 251, 16 to 251, 17 to 251, 18 to 251, 19 to 251, 20 to 251, 21 to 251, 22 to 251, 23 to 251, 24 to 251, 25 to 251, 26 to 251, 27 to 251, 28 to 251, 29 to 251, 30 to 251, 31 to 251, 32 to 251, 33 to 251, 34 to 251, 35 to 251, 36 to 251, 37 to 251, 38 to 251, 39 to 251, 40 to 251, 41 to 251, 41 to 251, 42 to 251, 43 to 251, 44 to 251, 45 to 251, 46 to 251, 47 to 251, 48 to 251, 49 to 251, 50 to 251, 51 to 251, 52 to 251, 53 to 251, 54 to 251, 55 to 251, 56 to 251, 57 to 251, 58 to 251, 59 to 251, 60 to 251, 61 to 251, 62 to 251, 63 to 251, 64 to 251, 65 to 251, 66 to 251, 67 to 251, 68 to 251, 69 to 251, 70 to 251, 71 to 251, 72 to 251, 73 to 251, 74 to 251, 75 to 251, 76 to 251, 77 to 251, 78 to 251, 79 to 251, 80 to 251, 81 to 251, 82 to 251, 83 to 251, 84 to 251, 85 to 251, 86 to 251, 87 to 251, 88 to 251, 89 to 251, 90 to 251, 91 to 251, 92 to 251, 93 to 251, 94 to 251, 95 to 251, 96 to 251, 97 to 251, 98 to 251, 99 to 251, 100 to 251, 101 to 251, 102 to 251, 103 to 251, 104 to 251, 105 to 251, 106 to 251, 107 to 251, 108 to 251, 109 to 251, 110 to 251, 111 to 251, 112 to 251, 113 to 251, 114 to 251, 115 to 251, 116 to 251, 117 to 251, 118 to 251, 119 to 251, 120 to 251, 121 to 251, 122 to 251, 123 to 251, 124 to 251, 125 to 251, 126 to 251, 127 to 251, 128 to 251, 129 to 251, 130 to 251, 131 to 251, 133 to 251, 134 to 251, and 134 to 251 of SEQ ID NO:20. Polynucleotides encoding these polypeptides are also encompassed by the invention.

*Sub C8* As mentioned above, even if deletion of one or more amino acids from the N-terminus of a polypeptide results in modification of loss of one or more biological functions of the polypeptide, other biological activities may still be retained. Thus, the ability of the shortened TNF-gamma-alpha mutein to induce and/or bind to antibodies which recognize the full-length or mature form of the polypeptide generally will be retained when less than the majority of the residues of the full-length or mature polypeptide are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a TNF-gamma-alpha mutein with a large number of deleted N-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six TNF-gamma-alpha amino acid residues may often evoke an immune response.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the amino terminus of the predicted mature amino acid

sequence of the TNF-gamma-alpha shown in Figures 1A and 1B (SEQ ID NO:2), up to the phenylalanine residue at position number 169 of the sequence shown in Figures 1A and 1B (which corresponds to position number 142 of SEQ ID NO:2) and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising the amino acid sequence of residues  $n^3$ -174 of the sequence shown in Figures 1A and 1B ( $n^3$ -147 of SEQ ID NO:2), where  $n^3$  is an integer in the range of 1 to 169, and 170 is the position of the first residue from the N-terminus of the complete TNF-gamma-alpha polypeptide believed to be required for at least immunogenic activity of the TNF-gamma-alpha polypeptide.

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues of R-2 to L-174; R-3 to L-174; F-4 to L-174; L-5 to L-174; S-6 to L-174; K-7 to L-174; V-8 to L-174; Y-9 to L-174; S-10 to L-174; F-11 to L-174; P-12 to L-174; M-13 to L-174; R-14 to L-174; K-15 to L-174; L-16 to L-174; I-17 to L-174; L-18 to L-174; F-19 to L-174; L-20 to L-174; V-21 to L-174; F-22 to L-174; P-23 to L-174; V-24 to L-174; V-25 to L-174; R-26 to L-174; Q-27 to L-174; T-28 to L-174; P-29 to L-174; T-30 to L-174; Q-31 to L-174; H-32 to L-174; F-33 to L-174; K-34 to L-174; N-35 to L-174; Q-36 to L-174; F-37 to L-174; P-38 to L-174; A-39 to L-174; L-40 to L-174; H-41 to L-174; W-42 to L-174; E-43 to L-174; H-44 to L-174; E-45 to L-174; L-46 to L-174; G-47 to L-174; L-48 to L-174; A-49 to L-174; F-50 to L-174; T-51 to L-174; K-52 to L-174; N-53 to L-174; R-54 to L-174; M-55 to L-174; N-56 to L-174; Y-57 to L-174; T-58 to L-174; N-59 to L-174; K-60 to L-174; F-61 to L-174; L-62 to L-174; L-63 to L-174; I-64 to L-174; P-65 to L-174; E-66 to L-174; S-67 to L-174; G-68 to L-174; D-69 to L-174; Y-70 to L-174; F-71 to L-174; I-72 to L-174; Y-73 to L-174; S-74 to L-174; Q-75 to L-174; V-76 to L-174; T-77 to L-174; F-78 to L-174; R-79 to L-174; G-80 to L-174; M-81 to L-174; T-82 to L-174; S-83 to L-174; E-84 to L-174; C-85 to L-174; S-86 to L-174; E-87 to L-174; I-88 to L-174; R-89 to L-174; Q-90 to L-174; A-91 to L-174; G-92 to L-174; R-93 to L-174; P-94 to L-174; N-95 to L-174; K-96 to L-174; P-97 to L-174; D-98 to L-174; S-99 to L-174; I-100 to L-174; T-101 to L-174; V-102 to L-174; V-103 to L-174; I-104 to L-174; T-105 to L-174; K-106 to L-174; V-107 to L-174; T-108 to L-174; D-109 to L-174; S-110 to L-174; Y-111 to L-174; P-112 to L-174; E-113 to L-174; P-114 to L-174; T-115 to L-174; Q-116 to L-174; L-117 to L-174; L-118 to L-174; M-119 to L-174; G-120 to L-174; T-121 to L-174; K-122 to L-174; S-123 to L-174; V-124 to L-174; C-125 to L-174; E-126 to L-174; V-127 to L-174; G-128 to L-174; S-129 to L-174; N-130 to L-174; W-131 to L-174; F-132 to L-174; Q-133 to L-174; P-134 to L-174; I-135 to L-174; Y-136 to L-174; L-137 to L-174; G-138 to L-174; A-139 to L-174;

M-140 to L-174; F-141 to L-174; S-142 to L-174; L-143 to L-174; Q-144 to L-174; E-145 to L-174; G-146 to L-174; D-147 to L-174; K-148 to L-174; L-149 to L-174; M-150 to L-174; V-151 to L-174; N-152 to L-174; V-153 to L-174; S-154 to L-174; D-155 to L-174; I-156 to L-174; S-157 to L-174; L-158 to L-174; V-159 to L-174; 5 D-160 to L-174; Y-161 to L-174; T-162 to L-174; K-163 to L-174; E-164 to L-174; D-165 to L-174; K-166 to L-174; T-167 to L-174; F-168 to L-174; and F-169 to L-174 of the TNF-gamma-alpha sequence shown in Figures 1A and 1B (the TNF-gamma-alpha amino acid sequence shown in Figures 1A and 1B is identical to that in SEQ ID NO:2, however, the numbering scheme differs between the two; the 10 numbering of the above amino acid residues in this case reflects that of Figures 1A and 1B). Polynucleotides encoding these polypeptides are also encompassed by the invention.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the amino terminus of the predicted mature amino acid 15 sequence of the TNF-gamma-beta shown in SEQ ID NO:20, up to the phenylalanine residue at position number 246 and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising the amino acid sequence of residues  $n^4$ -251 of SEQ ID NO:20, where  $n^4$  is an integer in the range of 2 to 246, and 247 is the position of the first residue from the N-terminus of the 20 complete TNF-gamma-beta polypeptide believed to be required for at least immunogenic activity of the TNF-gamma-beta protein.

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues of A-2 to L-251; E-3 to L-251; D-4 to L-251; L-5 to L-251; G-6 to L-251; 25 L-7 to L-251; S-8 to L-251; F-9 to L-251; G-10 to L-251; E-11 to L-251; T-12 to L-251; A-13 to L-251; S-14 to L-251; V-15 to L-251; E-16 to L-251; M-17 to L-251; L-18 to L-251; P-19 to L-251; E-20 to L-251; H-21 to L-251; G-22 to L-251; S-23 to L-251; C-24 to L-251; R-25 to L-251; P-26 to L-251; K-27 to L-251; A-28 to L-251; R-29 to L-251; S-30 to L-251; S-31 to L-251; S-32 to L-251; A-33 to L-251; R-34 to 30 L-251; W-35 to L-251; A-36 to L-251; L-37 to L-251; T-38 to L-251; C-39 to L-251; C-40 to L-251; L-41 to L-251; V-42 to L-251; L-43 to L-251; L-44 to L-251; P-45 to L-251; F-46 to L-251; L-47 to L-251; A-48 to L-251; G-49 to L-251; L-50 to L-251; T-51 to L-251; T-52 to L-251; Y-53 to L-251; L-54 to L-251; L-55 to L-251; V-56 to L-251; S-57 to L-251; Q-58 to L-251; L-59 to L-251; R-60 to L-251; A-61 to L-251; 35 Q-62 to L-251; G-63 to L-251; E-64 to L-251; A-65 to L-251; C-66 to L-251; V-67 to L-251; Q-68 to L-251; F-69 to L-251; Q-70 to L-251; A-71 to L-251; L-72 to L-251; K-73 to L-251; G-74 to L-251; Q-75 to L-251; E-76 to L-251; F-77 to L-251; A-78 to

L-251; P-79 to L-251; S-80 to L-251; H-81 to L-251; Q-82 to L-251; Q-83 to L-251; V-84 to L-251; Y-85 to L-251; A-86 to L-251; P-87 to L-251; L-88 to L-251; R-89 to L-251; A-90 to L-251; D-91 to L-251; G-92 to L-251; D-93 to L-251; K-94 to L-251; P-95 to L-251; R-96 to L-251; A-97 to L-251; H-98 to L-251; L-99 to L-251; T-100  
5 to L-251; V-101 to L-251; V-102 to L-251; R-103 to L-251; Q-104 to L-251; T-105 to L-251; P-106 to L-251; T-107 to L-251; Q-108 to L-251; H-109 to L-251; F-110 to L-251; K-111 to L-251; N-112 to L-251; Q-113 to L-251; F-114 to L-251; P-115 to L-251; A-116 to L-251; L-117 to L-251; H-118 to L-251; W-119 to L-251; E-120 to L-251; H-121 to L-251; E-122 to L-251; L-123 to L-251; G-124 to L-251; L-125 to  
10 L-251; A-126 to L-251; F-127 to L-251; T-128 to L-251; K-129 to L-251; N-130 to L-251; R-131 to L-251; M-132 to L-251; N-133 to L-251; Y-134 to L-251; T-135 to L-251; N-136 to L-251; K-137 to L-251; F-138 to L-251; L-139 to L-251; L-140 to L-251; I-141 to L-251; P-142 to L-251; E-143 to L-251; S-144 to L-251; G-145 to L-251; D-146 to L-251; Y-147 to L-251; F-148 to L-251; I-149 to L-251; Y-150 to  
15 L-251; S-151 to L-251; Q-152 to L-251; V-153 to L-251; T-154 to L-251; F-155 to L-251; R-156 to L-251; G-157 to L-251; M-158 to L-251; T-159 to L-251; S-160 to L-251; E-161 to L-251; C-162 to L-251; S-163 to L-251; E-164 to L-251; I-165 to L-251; R-166 to L-251; Q-167 to L-251; A-168 to L-251; G-169 to L-251; R-170 to L-251; P-171 to L-251; N-172 to L-251; K-173 to L-251; P-174 to L-251; D-175 to  
20 L-251; S-176 to L-251; I-177 to L-251; T-178 to L-251; V-179 to L-251; V-180 to L-251; I-181 to L-251; T-182 to L-251; K-183 to L-251; V-184 to L-251; T-185 to L-251; D-186 to L-251; S-187 to L-251; Y-188 to L-251; P-189 to L-251; E-190 to L-251; P-191 to L-251; T-192 to L-251; Q-193 to L-251; L-194 to L-251; L-195 to L-251; M-196 to L-251; G-197 to L-251; T-198 to L-251; K-199 to L-251; S-200 to  
25 L-251; V-201 to L-251; C-202 to L-251; E-203 to L-251; V-204 to L-251; G-205 to L-251; S-206 to L-251; N-207 to L-251; W-208 to L-251; F-209 to L-251; Q-210 to L-251; P-211 to L-251; I-212 to L-251; Y-213 to L-251; L-214 to L-251; G-215 to L-251; A-216 to L-251; M-217 to L-251; F-218 to L-251; S-219 to L-251; L-220 to L-251; Q-221 to L-251; E-222 to L-251; G-223 to L-251; D-224 to L-251; K-225 to  
30 L-251; L-226 to L-251; M-227 to L-251; V-228 to L-251; N-229 to L-251; V-230 to L-251; S-231 to L-251; D-232 to L-251; I-233 to L-251; S-234 to L-251; L-235 to L-251; V-236 to L-251; D-237 to L-251; Y-238 to L-251; T-239 to L-251; K-240 to L-251; E-241 to L-251; D-242 to L-251; K-243 to L-251; T-244 to L-251; F-245 to L-251; and F-246 to L-251 of the TNF-gamma-beta sequence shown in SEQ ID  
35 NO:20. Polynucleotides encoding these polypeptides are also encompassed by the invention.

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Similarly, many examples of biologically functional C-terminal deletion muteins are known. For instance, Interferon gamma shows up to ten times higher activities by deleting 8 to 10 amino acid residues from the carboxy terminus of the protein (Dobeli, *et al.*, *J. Biotechnology* **7**:199-216 (1988)). Further, several

5 investigators have reported biologically inactive TNF- $\alpha$  muteins in which as few as two amino acids had been removed from the C-terminus (Carlino, J. A., *et al.*, *J. Biol. Chem.* **262**:958-961 (1987); Creasey, A. A., *et al.*, *Cancer Res.* **47**:145-149 (1987); Sidhu, R. S. and Bollon, A. P. *Anticancer Res.* **9**:1569-1576 (1989); Gase, K., *et al.*, *Immunology* **71**:368-371 (1990)).

10 In the present case, since the proteins of the invention are members of the TNF polypeptide family, deletions of C-terminal amino acids up to the leucine at position 146 of SEQ ID NO:2 (which corresponds to the leucine at position 250 of SEQ ID NO:20) may retain some biological activity such as regulation of growth and differentiation of many types of hematopoietic and endothelial cells. Polypeptides

15 having further C-terminal deletions including the leucine residue at position 146 of SEQ ID NO:2 (or the leucine residue at position 250 of SEQ ID NO:20) would not be expected to retain such biological activities because it is known that this residue in TNF-related polypeptides is in the beginning of the conserved domain required for biological activities.

20 However, even if deletion of one or more amino acids from the C-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature form of the protein generally will be retained when less than the majority of the

25 residues of the complete or mature protein are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.

In additional embodiments, the present invention further provides polypeptides

30 having one or more residues removed from the carboxy terminus of the amino acid sequence of the TNF- $\gamma$ - $\alpha$  shown in SEQ ID NO:2, up to the leucine residue at position 146 of SEQ ID NO:2, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides having the amino acid sequence of residues  $-27-m^1$  of the amino acid sequence in SEQ ID NO:2, where  $m^1$  is any

35 integer in the range of 146 to 147, and residue 146 is the position of the first residue from the C-terminus of the complete TNF- $\gamma$ - $\alpha$  polypeptide (shown in SEQ



ID NO:2) believed to be required for regulation of growth and differentiation of many types of hematopoietic and endothelial cells by the TNF-gamma-alpha polypeptide.

More in particular, the invention provides polynucleotides encoding polypeptides having the amino acid sequence of residues -27-146 and -27-147 of SEQ ID NO:2. Polynucleotides encoding these polypeptides also are provided.

The present invention also provides polypeptides having one or more residues removed from the carboxy terminus of the amino acid sequence of the TNF-gamma-beta shown in SEQ ID NO:20, up to the leucine residue at position 250 of SEQ ID NO:20, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides having the amino acid sequence of residues 1-m<sup>2</sup> of the amino acid sequence in SEQ ID NO:20, where m<sup>2</sup> is any integer in the range of 250 to 251, and residue 249 is the position of the first residue from the C-terminus of the complete TNF-gamma-beta polypeptide (shown in SEQ ID NO:20) believed to be required for regulation of growth and differentiation of many types of hematopoietic and endothelial cells.

More in particular, the invention provides polynucleotides encoding polypeptides having the amino acid sequence of residues 1-250 and 1-251 of SEQ ID NO:20. Polynucleotides encoding these polypeptides also are provided.

The invention also provides polypeptide fragments comprising, or alternatively consisting of, one or more amino acids deleted from both the amino and the carboxyl termini of TNF-gamma-alpha, which may be described generally as having residues n<sup>1</sup>-m<sup>1</sup> of SEQ ID NO:2, where n and m are integers as described above. The invention further provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini of TNF-gamma-beta, which may be described generally as having residues n<sup>2</sup>-m<sup>2</sup> of SEQ ID NO:20, where n<sup>2</sup> and m<sup>2</sup> are integers as described above.

As mentioned above, even if deletion of one or more amino acids from the C-terminus of a polypeptide results in modification of loss of one or more biological functions of the polypeptide, other biological activities may still be retained. Thus, the ability of the shortened TNF-gamma-alpha mutein to induce and/or bind to antibodies which recognize the full-length or mature of the polypeptide generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a full-length polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a TNF-gamma-alpha mutein with a large number of deleted C-terminal amino acid residues may retain some biological or immunogenic activities.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the carboxy terminus of the amino acid sequence of the TNF-gamma-alpha shown in Figures 1A and 1B (or in SEQ ID NO:2), up to the serine residue at position number 6 in Figures 1A and 1B (or -22 in SEQ ID NO:2), and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising the amino acid sequence of residues 1-m<sup>3</sup> of SEQ ID NO:2, where m<sup>3</sup> is an integer in the range of 6 to 174, and 6 is the position of the first residue from the C-terminus of the complete TNF-gamma-alpha polypeptide believed to be required for at least immunogenic activity of TNF-gamma-alpha.

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues M-1 to L-173; M-1 to F-172; M-1 to A-171; M-1 to G-170; M-1 to F-169; M-1 to F-168; M-1 to T-167; M-1 to K-166; M-1 to D-165; M-1 to E-164; M-1 to K-163; M-1 to T-162; M-1 to Y-161; M-1 to D-160; M-1 to V-159; M-1 to L-158; M-1 to S-157; M-1 to I-156; M-1 to D-155; M-1 to S-154; M-1 to V-153; M-1 to N-152; M-1 to V-151; M-1 to M-150; M-1 to L-149; M-1 to K-148; M-1 to D-147; M-1 to G-146; M-1 to E-145; M-1 to Q-144; M-1 to L-143; M-1 to S-142; M-1 to F-141; M-1 to M-140; M-1 to A-139; M-1 to G-138; M-1 to L-137; M-1 to Y-136; M-1 to I-135; M-1 to P-134; M-1 to Q-133; M-1 to F-132; M-1 to W-131; M-1 to N-130; M-1 to S-129; M-1 to G-128; M-1 to V-127; M-1 to E-126; M-1 to C-125; M-1 to V-124; M-1 to S-123; M-1 to K-122; M-1 to T-121; M-1 to G-120; M-1 to M-119; M-1 to L-118; M-1 to L-117; M-1 to Q-116; M-1 to T-115; M-1 to P-114; M-1 to E-113; M-1 to P-112; M-1 to Y-111; M-1 to S-110; M-1 to D-109; M-1 to T-108; M-1 to V-107; M-1 to K-106; M-1 to T-105; M-1 to I-104; M-1 to V-103; M-1 to V-102; M-1 to T-101; M-1 to I-100; M-1 to S-99; M-1 to D-98; M-1 to P-97; M-1 to K-96; M-1 to N-95; M-1 to P-94; M-1 to R-93; M-1 to G-92; M-1 to A-91; M-1 to Q-90; M-1 to R-89; M-1 to I-88; M-1 to E-87; M-1 to S-86; M-1 to C-85; M-1 to E-84; M-1 to S-83; M-1 to T-82; M-1 to M-81; M-1 to G-80; M-1 to R-79; M-1 to F-78; M-1 to T-77; M-1 to V-76; M-1 to Q-75; M-1 to S-74; M-1 to Y-73; M-1 to I-72; M-1 to F-71; M-1 to Y-70; M-1 to D-69; M-1 to G-68; M-1 to S-67; M-1 to E-66; M-1 to P-65; M-1 to I-64; M-1 to L-63; M-1 to L-62; M-1 to F-61; M-1 to K-60; M-1 to N-59; M-1 to T-58; M-1 to Y-57; M-1 to N-56; M-1 to M-55; M-1 to R-54; M-1 to N-53; M-1 to K-52; M-1 to T-51; M-1 to F-50; M-1 to A-49; M-1 to L-48; M-1 to G-47; M-1 to L-46; M-1 to E-45; M-1 to H-44; M-1 to E-43; M-1 to W-42; M-1 to H-41; M-1 to L-40; M-1 to A-39; M-1 to P-38; M-1 to F-37; M-1 to

Q-36; M-1 to N-35; M-1 to K-34; M-1 to F-33; M-1 to H-32; M-1 to Q-31; M-1 to T-30; M-1 to P-29; M-1 to T-28; M-1 to Q-27; M-1 to R-26; M-1 to V-25; M-1 to V-24; M-1 to P-23; M-1 to F-22; M-1 to V-21; M-1 to L-20; M-1 to F-19; M-1 to L-18; M-1 to I-17; M-1 to L-16; M-1 to K-15; M-1 to R-14; M-1 to M-13; M-1 to P-12; M-1 to F-11; M-1 to S-10; M-1 to Y-9; M-1 to V-8; M-1 to K-7; and M-1 to S-6 of the sequence of the TNF-gamma-alpha sequence shown in Figures 1A and 1B (the TNF-gamma-alpha amino acid sequence shown in Figures 1A and 1B is identical to that in SEQ ID NO:2, however, the numbering scheme differs between the two; the numbering of the above amino acid residues in this case reflects that of Figures 1A and 1B). Polynucleotides encoding these polypeptides also are provided.

The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini of a TNF-gamma-alpha polypeptide, which may be described generally as having residues  $n^3$ - $m^3$  of SEQ ID NO:2, where  $n^3$  and  $m^3$  are integers as described above. Polynucleotides encoding the polypeptides are also encompassed by the invention.

The present invention further provides polypeptides having one or more residues deleted from the carboxy terminus of the amino acid sequence of the TNF-gamma-beta shown in SEQ ID NO:20, up to the glycine residue at position number 6, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising the amino acid sequence of residues 1- $m^4$  of SEQ ID NO:20, where  $m^4$  is an integer in the range of 6 to 250, and 6 is the position of the first residue from the C-terminus of the complete TNF-gamma-beta polypeptide believed to be required for at least immunogenic activity of the TNF-gamma-beta protein.

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues M-1 to L-250; M-1 to F-249; M-1 to A-248; M-1 to G-247; M-1 to F-246; M-1 to F-245; M-1 to T-244; M-1 to K-243; M-1 to D-242; M-1 to E-241; M-1 to K-240; M-1 to T-239; M-1 to Y-238; M-1 to D-237; M-1 to V-236; M-1 to L-235; M-1 to S-234; M-1 to I-233; M-1 to D-232; M-1 to S-231; M-1 to V-230; M-1 to N-229; M-1 to V-228; M-1 to M-227; M-1 to L-226; M-1 to K-225; M-1 to D-224; M-1 to G-223; M-1 to E-222; M-1 to Q-221; M-1 to L-220; M-1 to S-219; M-1 to F-218; M-1 to M-217; M-1 to A-216; M-1 to G-215; M-1 to L-214; M-1 to Y-213; M-1 to I-212; M-1 to P-211; M-1 to Q-210; M-1 to F-209; M-1 to W-208; M-1 to N-207; M-1 to S-206; M-1 to G-205; M-1 to V-204; M-1 to E-203; M-1 to C-202; M-1 to V-201; M-1 to S-200; M-1 to K-199; M-1 to T-198; M-1 to G-197; M-1 to M-196; M-1 to L-195; M-1 to L-194; M-1 to Q-193; M-1 to T-192; M-1 to P-191;

M-1 to E-190; M-1 to P-189; M-1 to Y-188; M-1 to S-187; M-1 to D-186; M-1 to  
 T-185; M-1 to V-184; M-1 to K-183; M-1 to T-182; M-1 to I-181; M-1 to V-180; M-1  
 to V-179; M-1 to T-178; M-1 to I-177; M-1 to S-176; M-1 to D-175; M-1 to P-174;  
 5 M-1 to K-173; M-1 to N-172; M-1 to P-171; M-1 to R-170; M-1 to G-169; M-1 to  
 A-168; M-1 to Q-167; M-1 to R-166; M-1 to I-165; M-1 to E-164; M-1 to S-163; M-1  
 to C-162; M-1 to E-161; M-1 to S-160; M-1 to T-159; M-1 to M-158; M-1 to G-157;  
 M-1 to R-156; M-1 to F-155; M-1 to T-154; M-1 to V-153; M-1 to Q-152; M-1 to  
 S-151; M-1 to Y-150; M-1 to I-149; M-1 to F-148; M-1 to Y-147; M-1 to D-146; M-1  
 to G-145; M-1 to S-144; M-1 to E-143; M-1 to P-142; M-1 to I-141; M-1 to L-140;  
 10 M-1 to L-139; M-1 to F-138; M-1 to K-137; M-1 to N-136; M-1 to T-135; M-1 to  
 Y-134; M-1 to N-133; M-1 to M-132; M-1 to R-131; M-1 to N-130; M-1 to K-129;  
 M-1 to T-128; M-1 to F-127; M-1 to A-126; M-1 to L-125; M-1 to G-124; M-1 to  
 L-123; M-1 to E-122; M-1 to H-121; M-1 to E-120; M-1 to W-119; M-1 to H-118;  
 M-1 to L-117; M-1 to A-116; M-1 to P-115; M-1 to F-114; M-1 to Q-113; M-1 to  
 15 N-112; M-1 to K-111; M-1 to F-110; M-1 to H-109; M-1 to Q-108; M-1 to T-107;  
 M-1 to P-106; M-1 to T-105; M-1 to Q-104; M-1 to R-103; M-1 to V-102; M-1 to  
 V-101; M-1 to T-100; M-1 to L-99; M-1 to H-98; M-1 to A-97; M-1 to R-96; M-1 to  
 P-95; M-1 to K-94; M-1 to D-93; M-1 to G-92; M-1 to D-91; M-1 to A-90; M-1 to  
 R-89; M-1 to L-88; M-1 to P-87; M-1 to A-86; M-1 to Y-85; M-1 to V-84; M-1 to  
 20 Q-83; M-1 to Q-82; M-1 to H-81; M-1 to S-80; M-1 to P-79; M-1 to A-78; M-1 to  
 F-77; M-1 to E-76; M-1 to Q-75; M-1 to G-74; M-1 to K-73; M-1 to L-72; M-1 to  
 A-71; M-1 to Q-70; M-1 to F-69; M-1 to Q-68; M-1 to V-67; M-1 to C-66; M-1 to  
 A-65; M-1 to E-64; M-1 to G-63; M-1 to Q-62; M-1 to A-61; M-1 to R-60; M-1 to  
 L-59; M-1 to Q-58; M-1 to S-57; M-1 to V-56; M-1 to L-55; M-1 to L-54; M-1 to  
 25 Y-53; M-1 to T-52; M-1 to T-51; M-1 to L-50; M-1 to G-49; M-1 to A-48; M-1 to  
 L-47; M-1 to F-46; M-1 to P-45; M-1 to L-44; M-1 to L-43; M-1 to V-42; M-1 to  
 L-41; M-1 to C-40; M-1 to C-39; M-1 to T-38; M-1 to L-37; M-1 to A-36; M-1 to  
 W-35; M-1 to R-34; M-1 to A-33; M-1 to S-32; M-1 to S-31; M-1 to S-30; M-1 to  
 30 S-23; M-1 to G-22; M-1 to H-21; M-1 to E-20; M-1 to P-19; M-1 to L-18; M-1 to  
 M-17; M-1 to E-16; M-1 to V-15; M-1 to S-14; M-1 to A-13; M-1 to T-12; M-1 to  
 E-11; M-1 to G-10; M-1 to F-9; M-1 to S-8; M-1 to L-7; and M-1 to G-6 of the  
 sequence of the TNF-gamma-beta sequence shown in SEQ ID NO:20.  
 Polynucleotides encoding these polypeptides also are provided.

35 The invention also provides polypeptides having one or more amino acids  
 deleted from both the amino and the carboxyl termini of a TNF-gamma-beta  
 polypeptide, which may be described generally as having residues n<sup>4</sup>-m<sup>4</sup> of SEQ ID

NO:20, where  $n^4$  and  $m^4$  are integers as described above. Polynucleotides encoding these polypeptides are also encompassed by the invention.

Further embodiments of the invention are directed to polypeptide fragments comprising, or alternatively, consisting of, amino acids described by the general formula  $m^x$  to  $n^x$ , where  $m$  and  $n$  correspond to any one of the amino acid residues specified above for these symbols, respectively, and  $x$  represents any integer. Polynucleotides encoding these polypeptides are also encompassed by the invention.

Specific embodiments of the invention are directed to nucleotide sequences encoding a polypeptide consisting of a portion of the complete TNF-gamma-alpha amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 75927, where this portion excludes from 1 to about 62 amino acids from the amino terminus of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 75927, or about 1 amino acid from the carboxy terminus, or any combination of the above amino terminal and carboxy terminal deletions, of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 75927. Polynucleotides encoding all of the above deletion mutant polypeptide forms also are provided.

In another embodiment, the invention is directed to a nucleotide sequence encoding a polypeptide consisting of a portion of the complete TNF-gamma-beta amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 203055, where this portion excludes from 1 to about 134 amino acids from the amino terminus of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 203055, or excludes a number of amino acids from the amino terminus of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 203055 (where the number is selected from any integer from 1 to 134), or about 1 amino acid from the carboxy terminus, or any combination of the above amino terminal and carboxy terminal deletions, of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 203055. Polynucleotides encoding all of the above polypeptides are also encompassed by the invention.

The invention further provides an isolated TNF-gamma polypeptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of the full-length TNF-gamma-alpha polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 (i.e., positions -27 to 147 of SEQ ID NO:2); (b) the amino acid sequence of the full-length TNF-gamma-alpha polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 excepting the N-terminal methionine (i.e., positions -26 to 147 of SEQ ID NO:2); (c)

the amino acid sequence of the predicted mature TNF-gamma-alpha polypeptide having the amino acid sequence at positions 1-147 in SEQ ID NO:2 (d) the complete amino acid sequence encoded by the cDNA clone HUVEO91 contained in the ATCC Deposit No. 75927; (e) the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone contained in the ATCC Deposit No. 75927; and (f) the complete amino acid sequence of the predicted mature TNF-gamma polypeptide encoded by the cDNA clone HUVEO91 contained in the ATCC Deposit No. 75927. The polypeptides of the present invention also include polypeptides having an amino acid sequence at least 70% identical, at least 80% identical, more preferably at least 90% identical, and still more preferably 95%, 96%, 97%, 98% or 99% identical to those described in (a), (b), (c), (d), (e) or (f), above, or fragments thereof, as described herein.

The invention further provides an isolated TNF-gamma polypeptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of the full-length TNF-gamma-beta polypeptide having the complete amino acid sequence shown in SEQ ID NO:20 (i.e., positions 1 to 251 of SEQ ID NO:20); (b) the amino acid sequence of the full-length TNF-gamma-beta polypeptide having the complete amino acid sequence shown in SEQ ID NO:20 excepting the N-terminal methionine (i.e., positions 2 to 251 of SEQ ID NO:20); (c) the amino acid sequence of the predicted mature TNF-gamma-beta polypeptide having the amino acid sequence at positions 62-251 in SEQ ID NO:20; (d) the complete amino acid sequence encoded by the cDNA clone HEMCZ56 contained in the ATCC Deposit No. 203055; (e) the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone HEMCZ56 contained in the ATCC Deposit No. 203055; and (f) the complete amino acid sequence of the predicted mature TNF-gamma polypeptide encoded by the cDNA clone HEMCZ56 contained in the ATCC Deposit No. 203055. The polypeptides of the present invention also include polypeptides having an amino acid sequence at least 70% identical, at least 80% identical, more preferably at least 90% identical, and still more preferably 95%, 96%, 97%, 98% or 99% identical to those described in (a), (b), (c), (d), (e) or (f), above, or fragments thereof, as described herein. In specific embodiments, these polypeptides are at least 10 amino acids, at least 15 amino acids, at least 20 amino acids, at least 25 amino acids, at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a TNF-gamma polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference

sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the TNF-gamma polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figures 1A and B (SEQ ID NO:2), the amino acid sequence encoded by deposited cDNA clone HUVEO91, or fragments thereof, can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected

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by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination of whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of this embodiment. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence. For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

The polypeptides of the present invention also include the polypeptide of SEQ ID NO:20 (in particular the extracellular domain of the polypeptide) as well as



polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:20 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:20 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:20 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

Further polypeptides of the present invention include polypeptides have at least 70% similarity, at least 90% similarity, more preferably at least 95% similarity, and still more preferably at least 96%, 97%, 98% or 99% similarity to those polypeptides described herein. The polypeptides of the invention also comprise those which are at least 70% identical, at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptides disclosed herein. In specific embodiments, such polypeptides comprise at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide. By "% similarity" for two polypeptides is intended a similarity score produced by comparing the amino acid sequences of the two polypeptides using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) and the default settings for determining similarity. Bestfit uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2:482-489, 1981) to find the best segment of similarity between two sequences.

The TNF-gamma-alpha and TNF-gamma-beta polypeptides of the invention may be in monomers or multimers (i.e., dimers, trimers, tetramers and higher multimers). In specific embodiments, the polypeptides of the invention are monomers, dimers, trimers or tetramers. In additional embodiments, the multimers of the invention are at least dimers, at least trimers, or at least tetramers.

Multimers encompassed by the invention may be homomers or heteromers. As used herein, the term homomer, refers to a multimer containing only TNF-gamma-alpha and/or TNF-gamma-beta polypeptides of the invention (including TNF-gamma-alpha and/or TNF-gamma-beta fragments, variants, and fusion proteins, as described herein). These homomers may contain TNF-gamma-alpha and TNF-gamma-beta polypeptides having identical or different amino acid sequences. In a specific embodiment, a homomer of the invention is a multimer containing only

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TNF-gamma-alpha and/or TNF-gamma-beta polypeptides having an identical amino acid sequence. In another specific embodiment, a homomer of the invention is a multimer containing TNF-gamma-alpha and TNF-gamma-beta polypeptides having different amino acid sequences. In specific embodiments, the multimer of the invention is a homodimer (e.g., containing TNF-gamma-alpha polypeptides having identical or different amino acid sequences) or a homotrimer (e.g., containing TNF-gamma-alpha polypeptides having identical or different amino acid sequences). In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

As used herein, the term heteromer refers to a multimer containing heterologous polypeptides (i.e., polypeptides of a different protein) in addition to the TNF-gamma-alpha and TNF-gamma-beta polypeptides of the invention. In a specific embodiment, the multimer of the invention is a heterodimer, a heterotrimer, or a heterotetramer. In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

Multimers of the invention may be the result of hydrophobic, hydrophilic, ionic and/or covalent associations. Thus, in one embodiment, multimers of the invention, such as, for example, homodimers or homotrimers, are formed when polypeptides of the invention contact one another in solution. In another embodiment, heteromultimers of the invention, such as, for example, heterotrimers or heterotetramers, are formed when polypeptides of the invention contact antibodies to the polypeptides of the invention (including antibodies to the heterologous polypeptide sequence in a fusion protein of the invention) in solution. In other embodiments, multimers of the invention are formed by covalent interactions with and/or between the TNF-gamma-alpha and TNF-gamma-beta polypeptides of the invention. Such covalent interactions may involve one or more amino acid residues corresponding to

those recited in ~~SEQ ID NO: 2~~ or ~~SEQ ID NO: 20~~, or corresponding to one or more amino acid residues ~~encoded by the clone~~ *encoded by the clones HUVED91 and HEMC756, respectively*. Alternatively, such covalent interactions may involve one or more amino acid residues contained in the heterologous polypeptide sequence in a TNF-gamma-alpha and TNF-gamma-beta fusion protein, such as for example, heterologous sequence contained in a TNF-gamma-alpha-Fc fusion protein (as described herein), and heterologous sequence contained in a fusion with heterologous polypeptide sequence from another TNF family ligand/receptor member, such as, for example, osteoprotegerin, that is capable of forming covalently associated multimers.

The invention also encompasses fusion proteins in which the full length TNF-gamma polypeptide or fragment, variant, derivative, or analog thereof is fused to an

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unrelated protein. Fusion proteins of the invention may be constructed as direct fusion of TNF-gamma polypeptide (or fragment, variant, derivative, or analog) and a heterologous sequence, or may be constructed with a spacer or adapter region having one or more amino acids inserted between the two portions of the protein. Optionally,

5 the spacer region may encode a protease cleavage site. The precise site of the fusion is not critical and may be routinely varied by one skilled in the art in order to maximize binding characteristics and/or biological activity of the homologous and/or hereologous sequence(s). The fusion proteins of the invention can be routinely designed on the basis of the TNF-gamma nucleotide and polypeptide sequences

10 disclosed herein. For example, as one of skill in the art will appreciate, TNF-gamma-alpha and/or TNF-gamma-beta polypeptides and fragments (including epitope-bearing fragments) thereof described herein can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric (fusion) polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*.

15 This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EP A 394,827; Traunecker, *et al.*, *Nature* **331**:84-86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing

20 other molecules than the monomeric TNF-gamma protein or protein fragment alone (Fountoulakis, *et al.*, *J. Biochem.* **270**:3958-3964 (1995)). As an example, one such TNF-gamma-Fc fusion has been produced herein as described above. In other embodiments, the full length TNF-gamma polypeptide or fragment, variant, derivative, or analog thereof is fused to one or more other heterologous polypeptide

25 sequences that are capable of forming multimeric formations, such as, for example, the dimerization domain of osteoprotegrin (see, e.g., EP 0 721 983, U.S. Patent No. 5,478,925, and International Publication No. WO 98/49305, each of which is herein incorporated by reference in its entirety). Additional examples of TNF-gamma fusion proteins that are encompassed by the invention include, but are not limited to, fusion

30 of the TNF-gamma polypeptide sequence to any amino acid sequence that allows the fusion protein to be displayed on the cell surface; or fusions to an enzyme, fluorescent protein, or luminescent protein which provides a marker function.

Modifications of chimeric OPG polypeptides are encompassed by the invention and include post-translational modifications (e.g., N-linked or O-linked

35 carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a

Also provided by the invention are chemically modified derivatives of OPG which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U. S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g., EP 0 401 384, herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., *Exp. Hematol.* 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide)

5 molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective  
10 proteins chemically modified at the N-terminus modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing  
15 polymer is achieved.

The polypeptides of the present invention have uses which include, but are not limited to, molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art.

## 20 ***Functional Activities***

The functional activity of TNF-gamma polypeptides, and fragments, variants derivatives, and analogs thereof, can be assayed by various methods.

For example, in one embodiment where one is assaying for the ability to bind or compete with full-length TNF-gamma polypeptide for binding to anti-TNF-gamma  
25 antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or  
30 radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by  
35 detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labelled. Many means are known in

the art for detecting binding in an immunoassay and are within the scope of the present invention.

In another embodiment, where a TNF-ligand is identified, binding can be assayed, e.g., by means well-known in the art. In another embodiment, physiological correlates of TNF-gamma binding to its substrates (signal transduction) can be assayed.

In addition, assays described herein (see Examples 5, 6, and 9-15, and otherwise known in the art may routinely be applied to measure the ability of TNF-gamma polypeptides and fragments, variants derivatives and analogs thereof to elicit TNF-gamma related biological activity (e.g., to inhibit, or alternatively promote, cell proliferation, tumor formation, angiogenesis, NF-kB activation and cell adhesion *in vitro* or *in vivo*).

Other methods will be known to the skilled artisan and are within the scope of the invention.

### **Antibodies**

The present invention further relates to antibodies and T-cell antigen receptors (TCR) which specifically bind the polypeptides of the present invention. The antibodies of the present invention include IgG (including IgG1, IgG2, IgG3, and IgG4), IgA (including IgA1 and IgA2), IgD, IgE, or IgM, and IgY. As used herein, the term "antibody" (Ab) is meant to include whole antibodies, including single-chain whole antibodies, and antigen-binding fragments thereof. Most preferably the antibodies are human antigen binding antibody fragments of the present invention include, but are not limited to, Fab, Fab' and F(ab')<sub>2</sub>, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V<sub>L</sub> or V<sub>H</sub> domain. The antibodies may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, rabbit, goat, guinea pig, camel, horse, or chicken.

Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entire or partial of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are any combinations of variable region(s) and hinge region, CH1, CH2, and CH3 domains. The present invention further includes chimeric, humanized, and human monoclonal and polyclonal antibodies which specifically bind the polypeptides of the present invention. The present invention further includes antibodies which are anti-idiotypic to the antibodies of the present invention.

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The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for heterologous compositions, such as a heterologous polypeptide or solid support material. *See, e.g.*, WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, A. et al. (1991) J. Immunol. 147:60-69; US Patents 5,573,920, 4,474,893, 5,601,819, 4,714,681, 4,925,648; Kostelny, S.A. et al. (1992) J. Immunol. 148:1547-1553.

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention which are recognized or specifically bound by the antibody. The epitope(s) or polypeptide portion(s) may be specified as described herein, *e.g.*, by N-terminal and C-terminal positions, by size in contiguous amino acid residues, or listed in the Tables and Figures. Antibodies which specifically bind any epitope or polypeptide of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind polypeptides of the present invention, and allows for the exclusion of the same.

Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homolog of the polypeptides of the present invention are included. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. Further included in the present invention are antibodies which only bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified in terms of their binding affinity. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  $5 \times 10^{-6}M$ ,  $10^{-6}M$ ,  $5 \times 10^{-7}M$ ,  $10^{-7}M$ ,  $5 \times 10^{-8}M$ ,  $10^{-8}M$ ,  $5 \times 10^{-9}M$ ,  $10^{-9}M$ ,  $5 \times 10^{-10}M$ ,  $10^{-10}M$ ,  $5 \times 10^{-11}M$ ,  $10^{-11}M$ ,  $5 \times 10^{-12}M$ ,  $10^{-12}M$ ,  $5 \times 10^{-13}M$ ,  $10^{-13}M$ ,  $5 \times 10^{-14}M$ ,  $10^{-14}M$ ,  $5 \times 10^{-15}M$ , and  $10^{-15}M$ .

Antibodies of the present invention have uses that include, but are not limited to, methods known in the art to purify, detect, and target the polypeptides of the present invention including both *in vitro* and *in vivo* diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in

biological samples. *See, e.g.,* Harlow et al., *ANTIBODIES: A LABORATORY MANUAL*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) (incorporated by reference in the entirety).

The antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, or toxins. *See, e.g.*, WO 92/08495; WO 91/14438; WO 89/12624; US Patent 5,314,995; and EP 0 396 387.

The antibodies of the present invention may be prepared by any suitable method known in the art. For example, a polypeptide of the present invention or an antigenic fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. Monoclonal antibodies can be prepared using a wide of techniques known in the art including the use of hybridoma and recombinant technology. *See, e.g.*, Harlow et al., ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entirety).

Fab and F(ab')<sub>2</sub> fragments may be produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')<sub>2</sub> fragments).

Alternatively, antibodies of the present invention can be produced through the application of recombinant DNA technology or through synthetic chemistry using methods known in the art. For example, the antibodies of the present invention can be prepared using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of a phage particle which carries polynucleotide sequences encoding them. Phage with a desired binding property are selected from a repertoire or combinatorial antibody library (e.g. human or murine) by selecting directly with antigen, typically antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman U. et al. (1995) J. Immunol. Methods 182:41-



50; Ames, R.S. et al. (1995) J. Immunol. Methods 184:177-186; Kettleborough, C.A. et al. (1994) Eur. J. Immunol. 24:952-958; Persic, L. et al. (1997) Gene 187 9-18; Burton, D.R. et al. (1994) Advances in Immunology 57:191-280; PCT/GB91/01134; WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and US Patents 5,698,426, 5,223,409, 5,403,484, 5,580,717, 5,427,908, 5,750,753, 5,821,047, 5,571,698, 5,427,908, 5,516,637, 5,780,225, 5,658,727 and 5,733,743 (said references incorporated by reference in their entireties).

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host including mammalian cells, insect cells, plant cells, yeast, and bacteria. For example, techniques to recombinantly produce Fab, Fab' and F(ab')<sub>2</sub> fragments can also be employed using methods known in the art such as those disclosed in WO 92/22324; Mullinax, R.L. et al. (1992) BioTechniques 12(6):864-869; and Sawai, H. et al. (1995) AJRI 34:26-34; and Better, M. et al. (1988) Science 240:1041-1043 (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al. (1991) Methods in Enzymology 203:46-88; Shu, L. et al. (1993) PNAS 90:7995-7999; and Skerra, A. et al. (1988) Science 240:1038-1040. For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. Methods for producing chimeric antibodies are known in the art. *See e.g.*, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Gillies, S.D. et al. (1989) J. Immunol. Methods 125:191-202; and US Patent 5,807,715. Antibodies can be humanized using a variety of techniques including CDR-grafting (EP 0 239 400; WO 91/09967; US Patent 5,530,101; and 5,585,089), veneering or resurfacing (EP 0 592 106; EP 0 519 596; Padlan E.A., (1991) Molecular Immunology 28(4/5):489-498; Studnicka G.M. et al. (1994) Protein Engineering 7(6):805-814; Roguska M.A. et al. (1994) PNAS 91:969-973), and chain shuffling (US Patent 5,565,332). Human antibodies can be made by a variety of methods known in the art including phage display methods described above. *See also*, US Patents 4,444,887, 4,716,111, 5,545,806, and 5,814,318; and WO 98/46645 (said references incorporated by reference in their entireties).

Further included in the present invention are antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide of the present invention. The antibodies may be specific for antigens other than polypeptides of the present invention. For example, antibodies may be used to target the polypeptides of the present invention to particular cell types, either *in vitro* or *in vivo*, by fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to the polypeptides of the present invention may also be used in *in vitro* immunoassays and purification methods using methods known in the art. *See e.g.*, Harbor et al. *supra* and WO 93/21232; EP 0 439 095; Naramura, M. et al. (1994) Immunol. Lett. 39:91-99; US Patent 5,474,981; Gillies, S.O. et al. (1992) PNAS 89:1428-1432; Fell, H.P. et al. (1991) J. Immunol. 146:2446-2452 (said references incorporated by reference in their entireties).

The present invention further includes compositions comprising the polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the hinge region, CH1 domain, CH2 domain, and CH3 domain or any combination of whole domains or portions thereof. The polypeptides of the present invention may be fused or conjugated to the above antibody portions to increase the *in vivo* half life of the polypeptides or for use in immunoassays using methods known in the art. The polypeptides may also be fused or conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. *See e.g.*, US Patents 5,336,603, 5,622,929, 5,359,046, 5,349,053, 5,447,851, 5,112,946; EP 0 307 434, EP 0 367 166; WO 96/04388, WO 91/06570; Ashkenazi, A. et al. (1991) PNAS 88:10535-10539; Zheng, X.X. et al. (1995) J. Immunol. 154:5590-5600; and Vil, H. et al. (1992) PNAS 89:11337-11341 (said references incorporated by reference in their entireties).

The invention further relates to antibodies which act as agonists or antagonists of the polypeptides of the present invention. For example, the present invention includes antibodies which disrupt the receptor/ligand interactions with the polypeptides of the invention either partially or fully. Included are both receptor-

specific antibodies and ligand-specific antibodies. Included are receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. Also include are receptor-specific antibodies which

5 both prevent ligand binding and receptor activation. Likewise, included are neutralizing antibodies which bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included are antibodies which activate the receptor. These antibodies may act as agonists for

10 either all or less than all of the biological activities affected by ligand-mediated receptor activation. The antibodies may be specified as agonists or antagonists for biological activities comprising specific activities disclosed herein. The above antibody agonists can be made using methods known in the art. See e.g., WO 96/40281; US Patent 5,811,097; Deng, B. et al. (1998) *Blood* 92(6):1981-1988; Chen, Z. et al. (1998)

15 *Cancer Res.* 58(16):3668-3678; Harrop, J.A. et al. (1998) *J. Immunol.* 161(4):1786-1794; Zhu, Z. et al. (1998) *Cancer Res.* 58(15):3209-3214; Yoon, D.Y. et al. (1998) *J. Immunol.* 160(7):3170-3179; Prat, M. et al. (1998) *J. Cell. Sci.* 111(Pt2):237-247; Pitard, V. et al. (1997) *J. Immunol. Methods* 205(2):177-190; Liautard, J. et al. (1997) *Cytokine* 9(4):233-241; Carlson, N.G. et al. (1997) *J. Biol. Chem.*

20 272(17):11295-11301; Taryman, R.E. et al. (1995) *Neuron* 14(4):755-762; Muller, Y.A. et al. (1998) *Structure* 6(9):1153-1167; Bartunek, P. et al. (1996) *Cytokine* 8(1):14-20 (said references incorporated by reference in their entireties).

### ***Transgenics***

25 The polypeptides of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are

30 used to express polypeptides of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the transgene (i.e., polynucleotides of the invention) into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear

35 microinjection ((each of the following references is hereby incorporated by reference) Paterson et al., *Appl. Microbiol. Biotechnol.* 40:691-698 (1994); Carver et al., *Biotechnology (NY)* 11:1263-1270 (1993); Wright et al., *Biotechnology (NY)* 9:830-

834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines ((the following reference is hereby incorporated by reference) Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells ((each of the following  
5 references is hereby incorporated by reference) Thompson et al., Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun ((the following reference is hereby incorporated by reference) see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic  
10 pluripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer ((the following reference is hereby incorporated by reference) Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

15 Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence ((each of the following references is hereby incorporated by reference) Campell et al., Nature 380:64-66 (1996); Wilmut et al., Nature 385:810-813 (1997)).

20 The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, *i.e.*, mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and  
25 activated in a particular cell type by following, for example, the teaching of Lasko et al. ((the following reference is hereby incorporated by reference) Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the  
30 polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence  
35 of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. ((the following reference is hereby

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.

Transgenic and “knock-out” animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of TNF-gamma-alpha and/or TNF-gamma-beta polypeptides, studying conditions and/or disorders associated with aberrant TNF-gamma-alpha and/or TNF-gamma-beta expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

Endogenous gene expression can also be reduced by inactivating or "knocking out" the gene and/or its promoter using targeted homologous recombination. ((each of the following references is hereby incorporated by reference) *E.g.*, see Smithies et al.,  
35 Nature 317:230-234 (1985); Thomas & Capecchi, Cell 51:503-512 (1987); Thompson et al., Cell 5:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the

5 In another embodiment, techniques known in the art are used to generate knockouts in  
cells that contain, but do not express the gene of interest. Insertion of the DNA  
construct, via targeted homologous recombination, results in inactivation of the  
targeted gene. Such approaches are particularly suited in research and agricultural  
fields where modifications to embryonic stem cells can be used to generate animal  
offspring with an inactive targeted gene ((each of the following references is hereby  
10 incorporated by reference) *e.g.*, see Thomas & Capecchi 1987 and Thompson 1989,  
*supra*). However this approach can be routinely adapted for use in humans provided  
the recombinant DNA constructs are directly administered or targeted to the required  
site *in vivo* using appropriate viral vectors that will be apparent to those of skill in the  
15 art.

In further embodiments of the invention, cells that are genetically engineered to express the polypeptides of the invention, or alternatively, that are genetically engineered not to express the polypeptides of the invention (e.g., knockouts) are administered to a patient *in vivo*. Such cells may be obtained from the patient (i.e., animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (e.g., lymphocytes), adipocytes, muscle cells, endothelial cells etc. The cells are genetically engineered *in vitro* using recombinant DNA techniques to introduce the coding sequence of polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, e.g., by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the polypeptides of the invention. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, e.g., in the circulation, or intraperitoneally. Alternatively, the cells can be incorporated into a matrix and implanted in the body, e.g., genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. ((each of the following references is hereby incorporated

When the cells to be administered are non-autologous or non-MHC compatible  
5 cells, they can be administered using well known techniques which prevent the  
development of a host immune response against the introduced cells. For example,  
the cells may be introduced in an encapsulated form which, while allowing for an  
exchange of components with the immediate extracellular environment, does not allow  
the introduced cells to be recognized by the host immune system.

## Diagnosis

In particular, it is believed that certain tissues in mammals with cancer of the immune and circulatory systems express significantly reduced levels of the TNF-gamma-alpha and/or TNF-gamma-beta protein and mRNA encoding the TNF-gamma-alpha and/or TNF-gamma-beta protein when compared to a  
35 corresponding "standard" level. Further, it is believed that enhanced levels of the TNF-gamma-alpha and/or TNF-gamma-beta protein can be detected in certain body

fluids (e.g., sera, plasma, urine, and spinal fluid) from mammals with such a cancer when compared to sera from mammals of the same species not having the cancer.

Thus, the invention provides a diagnostic method useful during diagnosis of a immune and circulatory systems disorder, including cancers of these systems, which involves measuring the expression level of the gene encoding the TNF-gamma-alpha and/or TNF-gamma-beta protein in immune and circulatory systems tissue or other cells or body fluid from an individual and comparing the measured gene expression level with a standard TNF-gamma-alpha and/or TNF-gamma-beta gene expression level, whereby an increase or decrease in the gene expression level compared to the standard is indicative of an immune and circulatory systems disorder.

Where a diagnosis of a disorder in the immune and circulatory systems, including diagnosis of a tumor, has already been made according to conventional methods, the present invention is useful as a prognostic indicator, whereby patients exhibiting depressed TNF-gamma-alpha and/or TNF-gamma-beta gene expression will experience a worse clinical outcome relative to patients expressing the gene at a level nearer the standard level.

By "assaying the expression level of the gene encoding the TNF-gamma-alpha and/or TNF-gamma-beta protein" is intended qualitatively or quantitatively measuring or estimating the level of the TNF-gamma-alpha and/or TNF-gamma-beta protein or the level of the mRNA encoding the TNF-gamma-alpha and/or TNF-gamma-beta protein in a first biological sample either directly (e.g., by determining or estimating absolute protein level or mRNA level) or relatively (e.g., by comparing to the TNF-gamma-alpha and/or TNF-gamma-beta protein level or mRNA level in a second biological sample). Preferably, the TNF-gamma-alpha and/or TNF-gamma-beta protein level or mRNA level in the first biological sample is measured or estimated and compared to a standard TNF-gamma-alpha and/or TNF-gamma-beta protein level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having the disorder or being determined by averaging levels from a population of individuals not having a disorder of the immune and circulatory systems. As will be appreciated in the art, once a standard TNF-gamma-alpha and/or TNF-gamma-beta protein level or mRNA level is known, it can be used repeatedly as a standard for comparison.

By "biological sample" is intended any biological sample obtained from an individual, body fluid, cell line, tissue culture, or other source which contains TNF-gamma-alpha and/or TNF-gamma-beta protein or mRNA. As indicated, biological samples include body fluids (such as sera, plasma, urine, synovial fluid and spinal fluid) which contain free TNF-gamma-alpha and/or TNF-gamma-beta protein,



immune and circulatory systems tissue, and other tissue sources found to express complete or mature TNF-gamma-alpha and/or TNF-gamma-beta or a TNF-gamma-alpha and/or TNF-gamma-beta receptor. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. Where the  
 5 biological sample is to include mRNA, a tissue biopsy is the preferred source.

Total cellular RNA can be isolated from a biological sample using any suitable technique such as the single-step guanidinium-thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (*Anal. Biochem.* **162**:156-159 (1987)). Levels of mRNA encoding the TNF-gamma-alpha and/or TNF-gamma-beta protein  
 10 are then assayed using any appropriate method. These include Northern blot analysis, S1 nuclease mapping, the polymerase chain reaction (PCR), reverse transcription in combination with the polymerase chain reaction (RT-PCR), and reverse transcription in combination with the ligase chain reaction (RT-LCR).

Assaying TNF-gamma-alpha and/or TNF-gamma-beta protein levels in a  
 15 biological sample can occur using antibody-based techniques. For example, TNF-gamma-alpha and/or TNF-gamma-beta protein expression in tissues can be studied with classical immunohistological methods (Jalkanen, M., *et al.*, *J. Cell. Biol.* **101**:976-985 (1985); Jalkanen, M., *et al.*, *J. Cell. Biol.* **105**:3087-3096 (1987)). Other antibody-based methods useful for detecting TNF-gamma-alpha  
 20 and/or TNF-gamma-beta protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine ( $^{125}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{112}\text{In}$ ), and technetium ( $^{99\text{m}}\text{Tc}$ ), and fluorescent labels,  
 25 such as fluorescein and rhodamine, and biotin.

In addition to assaying TNF-gamma-alpha and/or TNF-gamma-beta protein levels in a biological sample obtained from an individual, TNF-gamma-alpha and/or TNF-gamma-beta protein can also be detected *in vivo* by imaging. Antibody labels or markers for *in vivo* imaging of TNF-gamma-alpha and/or TNF-gamma-beta protein  
 30 include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

35 A TNF-gamma-alpha and/or TNF-gamma-beta protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example,  $^{131}\text{I}$ ,  $^{112}\text{In}$ ,  $^{99\text{m}}\text{Tc}$ ), a radio-opaque

substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously or intraperitoneally) into the mammal to be examined for immune system disorder. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of  $^{99m}\text{Tc}$ . The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain TNF-gamma-alpha and/or TNF-gamma-beta protein. *In vivo* tumor imaging is described by Burchiel and coworkers (Chapter 13 in *Tumor Imaging: The Radiochemical Detection of Cancer*, Burchiel, S. W. and Rhodes, B. A., eds., Masson Publishing Inc. (1982)).

### **Treatment**

As noted above, TNF-gamma-alpha and/or TNF-gamma-beta polynucleotides and polypeptides are useful for diagnosis of conditions involving abnormally high or low expression of TNF-gamma-alpha and/or TNF-gamma-beta activities. Given the cells and tissues where TNF-gamma-alpha and/or TNF-gamma-beta is expressed as well as the activities modulated by TNF-gamma-alpha and/or TNF-gamma-beta, it is readily apparent that a substantially altered (increased or decreased) level of expression of TNF-gamma-alpha and/or TNF-gamma-beta in an individual compared to the standard or "normal" level produces pathological conditions related to the bodily system(s) in which TNF-gamma-alpha and/or TNF-gamma-beta is expressed and/or is active.

It is well-known in the art that, in addition to a specific cellular function, cellular receptor molecules may also often be exploited by a virus as a means of initiating entry into a potential host cell. For example, it was recently discovered by Wu and colleagues (*J. Exp. Med.* **185**:1681-1691 (1997)) that the cellular chemokine receptor CCR5 functions not only as a cellular chemokine receptor, but also as a receptor for macrophage-tropic human immunodeficiency virus (HIV)-1. In addition, RANTES, MIP-1a, and MIP-1b, which are agonists for the cellular chemokine receptor CCR5, inhibit entry of various strains of HIV-1 into susceptible cell lines (Cocchi, F., *et al.*, *Science* **270**:1811-1815 (1995)). Thus, the invention also provides a method of treating an individual exposed to, or infected with, a virus through the prophylactic or therapeutic administration of TNF-gamma-alpha and/or TNF-gamma-beta, or an agonist or antagonist thereof, to block or disrupt the interaction of a viral particle with the TNF-gamma-alpha and/or TNF-gamma-beta receptor and, as a result, block the initiation or continuation of viral infectivity.

The TNF-gamma-alpha and/or TNF-gamma-beta of the present invention binds to the TNF-gamma-alpha and/or TNF-gamma-beta receptor and, as such, is likely to block immuno- and endothelial cell-tropic viral infections. Expression patterns of the cDNA clone encoding the present invention suggests that this molecule is expressed primarily in endothelial cells and select hematopoietic tissues. When considered together, these observations suggest that agonists and antagonists, including a receptor, of TNF-gamma-alpha and/or TNF-gamma-beta may be useful as a method of blocking or otherwise regulating the infectivity of immunotropic viral infections. A non-limiting list of viruses which infect humans and can infect cells of the hematopoietic system includes such retroviruses as HIV-1, HIV-2, human T-cell lymphotropic virus (HTLV)-I, and HTLV-II, as well as other DNA and RNA viruses such as herpes simplex virus (HSV)-1, HSV-2, HSV-6, cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes samirii, adenoviruses, rhinoviruses, influenza viruses, reoviruses, and the like.

The ability of TNF-gamma-alpha and/or TNF-gamma-beta of the present invention, or agonists or antagonists thereof, to prophylactically or therapeutically block viral infection may be easily tested by the skilled artisan. For example, Simmons and coworkers (*Science* 276:276-279 (1997)) and Arenzana-Seisdedos and colleagues (*Nature* 383:400 (1996)) each outline a method of observing suppression of HIV-1 infection by an antagonist of the CCR5 chemokine receptor and of the CC chemokine RANTES, respectively, in cultured peripheral blood mononuclear cells. Cells are cultured and infected with a virus, HIV-1 in both cases noted above. An agonist or antagonist of the CC chemokine or its receptor is then immediately added to the culture medium. Evidence of the ability of the agonist or antagonist of the chemokine or cellular receptor is determined by evaluating the relative success of viral infection at 3, 6, and 9 days postinfection.

Administration of a pharmaceutical composition comprising an amount of an isolated TNF-gamma-alpha and/or TNF-gamma-beta, or an agonist or antagonist thereof, of the invention to an individual either infected with a virus or at risk for infection with a virus is performed as described below.

The present invention is also useful for diagnosis or treatment of various immune and circulatory system-related disorders in mammals, preferably humans. Such disorders include tumors (an incomplete list of human tumors includes breast cancer, colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, adenoma, and the like) and tumor metastasis, infections by bacteria,

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viruses, and other parasites, immunodeficiencies, inflammatory diseases, lymphadenopathy, autoimmune diseases, graft versus host disease, and any dysregulation of immune and circulatory systems cell function including, but not limited to, autoimmunity, arthritis, leukemias, lymphomas, immunosuppression, immunity, humoral immunity, inflammatory bowel disease, myelo suppression, and the like.

Since TNF-gamma has been shown to induce activation of cellular NF-kB and c-jun N-terminal kinase (JNK), it is also useful in therapeutically regulating such cellular and immune systemic disorders as tumors and tumor metastases, infections by bacteria, viruses, and other parasites, immunodeficiencies, inflammatory diseases, lymphadenopathy, autoimmune diseases, graft versus host disease, autoimmunity, arthritis, leukemias, lymphomas, immunosuppression, inflammatory bowel disease, myelosuppression, and related sequelae.

Since TNF-gamma-alpha and TNF-gamma-beta belong to the TNF superfamily, they also also modulate angiogenesis. In addition, since TNF-gamma-alpha and/or TNF-gamma-beta inhibit immune cell functions, it will have a wide range of anti-inflammatory activities. TNF-gamma-alpha and/or TNF-gamma-beta may be employed as an anti-neovascularizing agent to treat solid tumors by stimulating the invasion and activation of host defense cells, e.g., cytotoxic T-cells and macrophages and by inhibiting the angiogenesis of tumors. Those of skill in the art will recognize other non-cancer indications where blood vessel proliferation is not wanted. They may also be employed to enhance host defenses against resistant chronic and acute infections, for example, mycobacterial infections via the attraction and activation of microbicidal leukocytes. TNF-gamma-alpha and/or TNF-gamma-beta may also be employed to inhibit T-cell proliferation by the inhibition of IL-2 biosynthesis for the treatment of T-cell mediated auto-immune diseases and lymphocytic leukemias. TNF-gamma-alpha and/or TNF-gamma-beta may also be employed to stimulate wound healing, both via the recruitment of debris clearing and connective tissue promoting inflammatory cells. In this same manner, TNF-gamma-alpha and/or TNF-gamma-beta may also be employed to treat other fibrotic disorders, including liver cirrhosis, osteoarthritis and pulmonary fibrosis. TNF-gamma-alpha and/or TNF-gamma-beta also increases the presence of eosinophils which have the distinctive function of killing the larvae of parasites that invade tissues, as in schistosomiasis, trichinosis and ascariasis. TNF-gamma-alpha and/or TNF-gamma-beta may also be employed to regulate hematopoiesis, by regulating the activation and differentiation of various hematopoietic progenitor cells, for example, to release mature leukocytes from the bone marrow following

chemotherapy, i.e., in stem cell mobilization. TNF-gamma-alpha and/or TNF-gamma-beta may also be employed to treat sepsis.

In a similar fashion, TNF-gamma-alpha and/or TNF-gamma-beta may be used to treat rheumatoid arthritis (RA) by inhibiting the increase in angiogenesis or the increase in endothelial cell proliferation required to sustain an invading pannus in bone and cartilage as is often observed in RA. Endothelial cell proliferation is increased in the synovia of RA patients as compared to patients with osteoarthritis (OA) or unaffected individuals. Neovascularization is needed to sustain the increased mass of the invading pannus into bone and cartilage. Inhibition of angiogenesis is associated with a significant decrease in the severity of both early and chronic arthritis in animal models.

It will also be appreciated by one of ordinary skill that, since the TNF-gamma-alpha and/or TNF-gamma-beta proteins of the invention are members of the TNF family the mature secreted form of the protein may be released in soluble form from the cells which express TNF-gamma by proteolytic cleavage. Therefore, when TNF-gamma-alpha and/or TNF-gamma-beta mature form or soluble extracellular domain is added from an exogenous source to cells, tissues or the body of an individual, the protein will exert its physiological activities on its target cells of that individual. Also, cells expressing this type II transmembrane protein may be added to cells, tissues or the body of an individual and these added cells will bind to cells expressing receptor for TNF-gamma-alpha and/or TNF-gamma-beta, whereby the cells expressing TNF-gamma-alpha and/or TNF-gamma-beta can cause actions (e.g. regulation of endothelial cell growth and regulation) on the receptor-bearing target cells.

Therefore, it will be appreciated that conditions caused by a decrease in the standard or normal level of TNF-gamma-alpha and/or TNF-gamma-beta activities in an individual, particularly disorders of the immune and circulatory systems, can be treated by administration of TNF-gamma-alpha and/or TNF-gamma-beta polypeptide (in the form of the mature protein). Thus, the invention also provides a method of treatment of an individual in need of an increased level of TNF-gamma-alpha and/or TNF-gamma-beta activity comprising administering to such an individual a pharmaceutical composition comprising an amount of an isolated TNF-gamma-alpha and/or TNF-gamma-beta polypeptide of the invention, particularly a mature form of the TNF-gamma-alpha and/or TNF-gamma-beta protein of the invention, effective to increase the TNF-gamma-alpha and/or TNF-gamma-beta activity level in such an individual.

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The TNF-gamma polypeptide may also be employed to treat autoimmune diseases such as Type I diabetes by enhancing the T-cell proliferative response.

Table 2  
Summary of TNF-gamma activity

5		Source				
	<u>Cell lines</u>	<u>and type</u>	<u>Cytotoxicity</u>	<u>Proliferation</u>	<u>Differentiation</u>	<u>Adhesion</u>
		mouse				
	L929	fibroblast	+	-	-	-
10		mouse				
	WEHI 164	fibrosarcoma	+++	-	-	-
		rat kidney				
	NRK-54E	epithelial-like	+	-	-	-
		human				
15	THP-1	monocytic	+	-	++	++
		leukemia				
		human				
	HL-60	promyelocytic	-	-	-	++
		leukemia				
20		human				
	Raji	Burkitt	-	-	-	-
		lymphoma				
		human				
	Jurkat	T-cell	++	-	-	-
25		lymphoma				
	Primary	HUVEC	-	++	-	?
		human arterial				
	Primary	endothelial	+	++	-	?
		human				
30	A-431	epidermoid	++	-	-	-
		carcinoma				
		human				
	293	embryonal	-	++	-	-
		kidney				

Legend: \* At high dose only. The numbers of "+" indicate the relative level of activities. "-" indicates no detected activity at the concentration range tested.

TNF-gamma may be used to inhibit the proliferation of endothelial cells, for example, aortic endothelial cells. As illustrated in Figure 10, at concentrations of up to 10 µg/ml, TNF-gamma can nearly completely inhibit the growth of endothelial cells while having no apparent effect on the growth of human breast cancer cells. As a result, TNF-gamma can be used to treat diseases and disorders in which inhibition of endothelial cell growth is advantageous. Inhibiting the growth of endothelial cells is desirable in the treatment of many types of cancers which depend on the generation of new blood vessels to support growth of the tumor. TNF-gamma can be used to inhibit the growth of such tumors by inhibiting the growth of endothelial cells which are a major cellular component of the blood vessel. Evidence of the ability of TNF-gamma to be effectively used in this fashion is presented in Figures 16A and 16B. These experiments are discussed in greater detail below.

In particular, TNF-gamma can be used to regulate endothelial cell growth when endothelial cells have already begun proliferating. Such a situation may when angiogenesis is occurring as a tumor-supporting mechanism as described above. Endogenous TNF-gamma expression is reduced in proliferating cultures of endothelial cells, whereas the expression of endogenous TNF-gamma is enhanced in quiescent endothelial cell cultures (Figure 4). As a result, it is preferable to use TNF-gamma of the present invention to reduce the rate of cell growth in cultures of proliferating endothelial cells, for example, during the increase in size of a tumor in a cancerous state.

TNF-gamma of the present invention has been used to reduce the formation of capillary-like tubular structures formed by endothelial cells *in vitro*. As illustrated in Figure 14, TNF-gamma of the present invention can be used to inhibit the formation of endothelial cells organized into capillary-like tubular structures in response to angiogenic factors such as FGF-2. Furthermore, isolated TNF-gamma of the present invention can also be used to inhibit the growth and organization of endothelial cells into capillary vessels in a modified chicken embryo chorioallantoic membrane (CAM), as shown in Figure 15. As a result, TNF-gamma of the present invention can be used to inhibit the formation of capillaries or capillary-like structures from endothelial cells *in vitro*.

TNF-gamma of the present invention can be used as an anti-cancer agent. As illustrated in Figure 16, TNF-gamma was used to inhibit the growth of human breast cancer cells in a xenograft tumor model. Despite the high tumorigenicity of these cells, treatment with TNF-gamma of the present invention resulted in a marked inhibition of the growth of the xenograft tumors. TNF-gamma, or a mutein thereof,



of the present invention, can be used to treat a number of cancers including, but not limited to, breast cancer, colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, adenoma, and the like.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to human disease.

This invention provides a method for identification of the receptor for TNF-gamma. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to TNF-gamma, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to TNF-gamma. Transfected cells which are grown on glass slides are exposed to labeled TNF-gamma. TNF-gamma can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, labeled TNF-gamma can be photoaffinity-linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing the TNF-gamma-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

TNF-gamma does not bind significantly to two soluble TNF receptors, sTNF-RI (p55) and sTNF-RII (p75). Accordingly, TNF-gamma may have activities inclusive of and additional to known TNF proteins (see Figure 13).

### **Formulations**

The TNF-gamma polypeptide composition will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical

condition of the individual patient (especially the side effects of treatment with TNF-gamma polypeptide alone), the site of delivery of the TNF-gamma polypeptide composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" of TNF-gamma polypeptide for purposes herein is thus determined by such considerations.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The TNF-gamma polypeptides and agonists and antagonists of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10 g/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 g/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

### **Gene Therapy**

The TNF-gamma polypeptides and agonists and antagonists which are polypeptides may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered



promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the b-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

5           The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, b-2, b-AM, PA12, T19-14X, VT-19-17-H2, CRE, b-CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described by Miller (*Human Gene Therapy* 1:5-14 (1990)), which is incorporated  
10       herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO<sub>4</sub> precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

15       The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

## Agonists and Antagonists - Assays and Molecules

25 This invention is also related to a method of screening compounds to identify those which mimic TNF-gamma (agonists) or prevent the effect of TNF-gamma (antagonists). An example of such a method takes advantage of the ability of TNF-gamma to significantly stimulate the proliferation of human endothelial cells in the presence of the comitogen Con A. Endothelial cells are obtained and cultured in  
30 96-well flat-bottomed culture plates (Costar, Cambridge, MA) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, UT), 1% L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, 0.1% gentamycin (Gibco Life Technologies, Grand Island, NY) in the presence of 2 g/ml of Con-A (Calbiochem, La Jolla, CA). Con-A, and the compound to be screened are added to a  
35 final volume of 0.2 ml. After 60 h at 37°C, cultures are pulsed with 1 Ci of [<sup>3</sup>H]thymidine (5 Ci/mmol; 1 Ci = 37 BGq; NEN) for 12-18 h and harvested onto glass fiber filters (PhD; Cambridge Technology, Watertown, MA). Mean

[<sup>3</sup>H]thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant [<sup>3</sup>H]-thymidine incorporation indicates stimulation of endothelial cell proliferation.

Alternatively, the response of a known second messenger system following  
5 interaction of TNF-gamma and receptor would be measured and compared in the presence or absence of the compound. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

To assay for antagonists, the assay described above is performed, however, in  
10 this assay TNF-gamma is added along with the compound to be screened and the ability of the compound to inhibit [<sup>3</sup>H]thymidine incorporation in the presence of TNF-gamma, indicates that the compound is an antagonist to TNF-gamma. Alternatively, TNF-gamma antagonists may be detected by combining TNF-gamma and a potential antagonist with membrane-bound TNF-gamma receptors or  
15 recombinant receptors under appropriate conditions for a competitive inhibition assay. TNF-gamma can be labeled, such as by radioactivity, such that the number of TNF-gamma molecules bound to the receptor can determine the effectiveness of the potential antagonist.

Alternatively, a mammalian cell or membrane preparation expressing the  
20 TNF-gamma receptor is incubated with labeled TNF-gamma in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured.

In another aspect of this embodiment the invention provides a method for  
25 identifying a receptor protein or other ligand-binding protein which binds specifically to a TNF-gamma polypeptide (e.g. DR3). For example, a cellular compartment, such as a membrane or a preparation thereof, may be prepared from a cell that expresses a molecule that binds TNF-gamma. The preparation is incubated with labeled TNF-gamma and complexes of TNF-gamma bound to the receptor or other binding protein are isolated and characterized according to routine methods known in the art.  
30 Alternatively, the TNF-gamma polypeptide may be bound to a solid support so that binding molecules solubilized from cells are bound to the column and then eluted and characterized according to routine methods.

In the assay of the invention for agonists or antagonists, a cellular  
35 compartment, such as a membrane or a preparation thereof, may be prepared from a cell that expresses a molecule that binds TNF-gamma, such as a molecule of a signaling or regulatory pathway modulated by TNF-gamma. The preparation is incubated with labeled TNF-gamma in the absence or the presence of a candidate

molecule which may be a TNF-gamma agonist or antagonist. The ability of the candidate molecule to bind the binding molecule is reflected in decreased binding of the labeled ligand. Molecules which bind gratuitously, i.e., without inducing the effects of TNF-gamma on binding the TNF-gamma binding molecule, are most likely to be good antagonists. Molecules that bind well and elicit effects that are the same as or closely related to TNF-gamma are agonists.

TNF-gamma-like effects of potential agonists and antagonists may be measured, for instance, by determining activity of a second messenger system following interaction of the candidate molecule with a cell or appropriate cell preparation, and comparing the effect with that of TNF-gamma or molecules that elicit the same effects as TNF-gamma. Second messenger systems that may be useful in this regard include but are not limited to AMP guanylate cyclase, ion channel or phosphoinositide hydrolysis second messenger systems.

Another example of an assay for TNF-gamma antagonists is a competitive assay that combines TNF-gamma and a potential antagonist with membrane-bound TNF-gamma receptor molecules or recombinant TNF-gamma receptor molecules under appropriate conditions for a competitive inhibition assay. TNF-gamma can be labeled, such as by radioactivity, such that the number of TNF-gamma molecules bound to a receptor molecule can be determined accurately to assess the effectiveness of the potential antagonist.

Potential antagonists include small organic molecules, peptides, polypeptides and antibodies that bind to a polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a binding molecule, such as a receptor molecule, without inducing TNF-gamma-induced activities, thereby preventing the action of TNF-gamma by excluding TNF-gamma from binding.

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed in a number of studies (for example, Okano, *J. Neurochem.* **56**:560 (1991); "Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression." CRC Press, Boca Raton, FL (1988)). Triple helix formation is discussed in a number of studies, as well (for instance, Lee, *et al.*, *Nucleic Acids Research* **6**:3073 (1979); Cooney, *et al.*, *Science* **241**:456 (1988); Dervan, *et al.*, *Science* **251**:1360 (1991)). The methods are based on binding of a polynucleotide to a complementary DNA or RNA. For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present

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invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of TNF-gamma. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into TNF-gamma polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of TNF-gamma protein.

Antibodies specific to TNF-gamma may be used as antagonists by binding to TNF-gamma and preventing it from binding to its receptor. Monoclonal antibodies are particularly effective in this regard. Antibodies specific to the TNF-gamma receptor, however, may mediate distinct cellular responses which tend to agonize the effects of TNF-gamma upon interaction with its receptor.

Potential TNF-gamma antagonists also include TNF-gamma mutants which bind to the TNF-gamma receptor and elicit no second messenger response to effectively block the receptor from its natural ligand. Specifically designed oligonucleotides and small molecules may also bind to the TNF-gamma receptor (e.g., DR3) and block it from TNF-gamma. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

Another potential TNF-gamma antagonist is a soluble form of the TNF-gamma receptor which binds to TNF-gamma and prevents it from interacting with membrane-bound TNF-gamma receptors. In this way, the receptors are not stimulated by TNF-gamma.

Another potential TNF-gamma antagonist is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of TNF-gamma. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the TNF-gamma polypeptide (Antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca

Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of TNF-gamma.

TNF-antagonists may also be employed to treat cachexia which is a lipid clearing defect resulting from a systemic deficiency of lipoprotein lipase which is suppressed by TNF-gamma. The TNF-gamma antagonists are also employed to treat cerebral malaria in which TNF-gamma appears to play a pathogenic role. The antagonists may also be employed to treat rheumatoid arthritis by inhibiting TNF-gamma induced production of inflammatory cytokines such as IL-1 in the synovial cells. When treating arthritis TNF-gamma is preferably injected intra-articularly.

The TNF-gamma antagonists may also be employed to prevent graft rejection by preventing the stimulation of the immune system in the presence of a graft by TNF-gamma.

15     The TNF-gamma antagonists may also be employed to treat osteoporosis since TNF-gamma may induce bone resorption.

Antagonists to TNF-gamma may also be employed as anti-inflammation agents since TNF-gamma mediates an enhanced inflammatory response.

The antagonists may also be used to treat endotoxic shock, also referred to as septic shock. This critical condition results from an exaggerated response to bacterial or other types of infection. This response leads to elevated levels of TNF-gamma which causes shock and tissue injury.

The present invention also relates to a diagnostic assay for detecting altered levels of TNF-gamma protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease, for example, tumors and cerebral malaria. Assays used to detect levels of TNF-gamma protein in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan, et al., Current Protocols in Immunology, 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the TNF-gamma antigen, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or in this example a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein like BSA. Next, the monoclonal antibody is incubated in the dish during



which time the monoclonal antibodies attach to any TNF-gamma proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to TNF-gamma. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of TNF-gamma protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to TNF-gamma are attached to a solid support and labeled TNF-gamma and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of TNF-gamma in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay TNF-gamma is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the TNF-gamma. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantitated.

## **Gene Mapping**

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the sequence is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Utilizing the techniques described above, the chromosomal location of TNF-gamma was determined with very high confidence to be 9q32. Previous chromosomal mapping studies have linked several developmental defects to loci in this area of chromosome 9.

### *Examples*

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures, unless otherwise stated. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37 C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., *et al.*, *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., *Virology*, 52:456-457 (1973).

**Example 1: Bacterial Expression and Purification of TNF-gamma**

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The DNA sequence encoding the full-length TNF-gamma ORF, ATCC Deposit No. 75927, was initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the TNF-gamma protein. Additional nucleotides corresponding to TNF-gamma were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer is shown as SEQ ID NO:13 and has the sequence 5'-GCG CGG ATC CAC CAT GAG ACG CTT TTT AAG CAA AGT C-3' which contains a *Bam* HI restriction enzyme site followed by the first 24 nucleotides of TNF-gamma coding sequence starting from the initiating methionine codon. The 3' sequence 5'-CGC GTC TAG ACT ATA GTA AGA AGG CTC CAA AGA AGG-3' (SEQ ID NO:14) contains sequences complementary to an *Xba* I site and 22 nucleotides of TNF-gamma. The restriction enzyme sites correspond to the restriction enzyme sites in the bacterial expression vector pQE-9 (Qiagen). pQE-9 was then digested with *Bam* HI and *Xba* I. The amplified sequences were ligated into pQE-9 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform an *E. coli* strain available from Qiagen under the trademark M15/rep 4 by the procedure described in Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the *lacI* repressor and also confers kanamycin resistance (*Kan<sup>r</sup>*). Transformants were identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.<sub>600</sub>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalactopyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the *lacI* repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 M Guanidine HCl (Guanidine HCl concentrations of greater than or equal to 2.5 M were empirically found to result in a higher level of purity of recovered recombinant protein). After clarification, solubilized TNF-gamma was

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purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). TNF-gamma was further purified by a second run on the Nickel-chelate column. TNF-gamma (90% pure) was eluted from the column in 6 M guanidine HCl pH 5.0 and for the purpose of renaturation was dialyzed in PBS buffer. The expression product was electrophoresed by SDS-PAGE, and the results may be seen in Figure 5 where lanes labeled "M" contain molecular weight markers; lane 1 is induced cell lysate; lane 2 is uninduced cell lysate; lane 3 is the TNF-gamma protein after two Nickel-chelate column purifications; lane 4 is the TNF-gamma protein after 1 column purification.

One of ordinary skill in the art will recognize that bacterial expression vectors other than pQE-9 may also be used to express TNF-gamma. One such preferred bacterial expression vector is pHE4-5. pHE4-5 may be obtained as pHE4-5/MPIFD23 plasmid DNA (this construct contains an unrelated insert which encodes an unrelated ORF). The pHE4-5/MPIFD23 plasmid was deposited with the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, on September 30, 1997 (Accession No. 209311). Using the *Nde* I and *Asp* 718 restriction sites flanking the unrelated MPIF ORF insert, one of ordinary skill in the art could easily use current molecular biological techniques to replace the unrelated ORF in the pHE4-5/MPIFD23 plasmid with the TNF-gamma ORF, or variations thereof, of the present invention.

**Example 2: Cloning and expression of TNF-gamma using the baculovirus expression system**

The DNA sequence encoding the full length TNF-gamma protein, ATCC No. 75927, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene: The 5' primer has the sequence 5'-GCG CGG ATC CAC CAT GAG ACG CTT TTT AAG CAA AGT C-3' (SEQ ID NO:15) and contains a *Bam* HI restriction enzyme site (in bold) followed by 24 nucleotides of the TNF-gamma gene (the initiation codon for translation "ATG" is underlined). The 3' primer has the sequence 5'-CGC GTC TAG ACT ATA GTA AGA AGG CTC CAA AGA AGG-3' (SEQ ID NO:16) and contains the cleavage site for the restriction endonuclease *Xba* I and 22 nucleotides complementary to the 3' non-translated sequence of the TNF-gamma gene. The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101 Inc., La Jolla, Ca.). The fragment was then digested with the endonucleases *Bam* HI and *Xba* I and then purified again on a 1% agarose gel. This fragment was designated F2.

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The vector pA2 (modification of pVL941 vector, discussed below) was used for the expression of the TNF-gamma protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, *A manual of methods for baculovirus vectors and insect cell culture procedures*, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases *Bam* HI and *Xba* I. The polyadenylation site of the simian virus SV40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from *E.coli* was inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences were flanked at both sides by viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could have been used in place of pA2, such as pRG1, pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., *Virology*, 170:31-39).

The plasmid was digested with the restriction enzymes *Bam* HI and *Xba* I and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). This vector DNA was designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. *E. coli* XL1 blue cells were then transformed. The sequence of the cloned fragment was confirmed by DNA sequencing.

5 µg of the plasmid pBac TNF-gamma was cotransfected with 1.0 µg of a commercially available linearized baculovirus ("BaculoGold baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987)).

1 µg of BaculoGold virus DNA and 5 µg of the plasmid pBac TNF-gamma were mixed in a sterile well of a microtiter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10 µl Lipofectin plus 90 µl Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1ml Grace' medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours, the transfection solution was removed from the plate and 1 ml of Grace's insect

medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days, the supernatant was collected and a plaque assay performed essentially as described by Summers and Smith (*supra*). As a modification, an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the virus was added to the cells, blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the recombinant baculovirus V-TNF-gamma at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 µCi of [<sup>35</sup>S]-methionine and 5 µCi [<sup>35</sup>S]-cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labeled proteins visualized by SDS-PAGE and autoradiography. Figure 6 illustrates a gel where lanes 1 and 3 are the medium of the TNF-gamma and control cultures and lanes 2 and 4 are the cell lysates of the TNF-gamma and the control cultures.

### **Example 3: Expression of Recombinant TNF-gamma in COS cells**

The expression of plasmid, TNF-gamma-HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron, and a polyadenylation site. A DNA fragment encoding the entire TNF-gamma precursor and a hemagglutinin antigen (HA) tag fused in frame to its 3' end was cloned into the polylinker region of the vector. Therefore, the recombinant protein expression is under the direction of the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A. Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37, 767). The fusion of HA tag

to our target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows: The DNA sequence encoding TNF-gamma, ATCC # 75927, was constructed by PCR on the original EST  
 5 cloned using two primers: the 5' primer (SEQ ID NO:15) contains a *Bam* HI site followed by 24 nucleotides of TNF-gamma coding sequence starting from the initiation codon; the 3' sequence 5'-CGC TCT AGA TCA AGC GTA GTC TGG GAC GTC GTA TGG ATA GTA AGA AGG CTC CAA AG-3' (SEQ ID NO:17) contains complementary sequences to *Xba* I site, translation stop codon, HA tag and  
 10 the last 18 nucleotides of the TNF-gamma coding sequence (not including the stop codon). Therefore, the PCR product contained a *Bam* HI site, TNF-gamma coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an *Xba* I site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, were digested with *Bam* HI and *Xba* I restriction enzymes and  
 15 ligated together. The ligation mixture was transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For  
 20 expression of the recombinant TNF-gamma, COS cells were transfected with the expression vector by DEAE-DEXTRAN method. (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the TNF-gamma HA protein was detected by radiolabelling and immunoprecipitation method. (E. Harlow, D. Lane, Antibodies: A  
 25 Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells were labeled for 8 hours with [<sup>35</sup>S]-S-cysteine two days post transfection. Culture media were then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5; Wilson, I. et al., *Id.* 37:767 (1984)). Both cell lysate and culture media were precipitated with an  
 30 HA-specific monoclonal antibody. Precipitated proteins were then analyzed on 15% SDS-PAGE gels.

#### ***Example 4: Expression pattern of TNF-gamma in human tissue***

RNA blot analysis was carried out to examine the levels of expression of  
 35 TNF-gamma in human tissues. Total cellular RNA samples were isolated with RNazol™ B system (Biotecx Laboratories, Inc. 6023 South Loop East, Houston, TX 77033). About 2 µg (for the RNA blot of Figure 3A) of total RNA isolated from each



human tissue specified was separated on 1% agarose-formaldehyde gel and blotted onto a nylon filter (Sambrook, Fritsch, and Maniatis, Molecular Cloning, Cold Spring Harbor Press, (1989)). The labeling reaction was done according to the Stratagene Prime-It kit with 50 ng TNF-gamma cDNA, to produce [<sup>32</sup>P]-labeled TNF-gamma cDNA. The labeled DNA was purified with a Select-G-50 column (5 Prime - 3 Prime, Inc. 5603 Arapahoe Road, Boulder, CO 80303). The filter was then hybridized with radioactive labeled full-length TNF-gamma gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub>, pH 7.4 and 7% SDS overnight at 65°C. After being washed twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS, the X-ray film was then exposed to the blot at -70°C overnight with an intensifying screen. The message RNA for TNF-gamma is abundant in kidney.

The same reaction was done to obtain the results shown in Figure 3B, with the exception that 10 µg poly-A RNA isolated from the indicated tissues was used. In this experiment, the messenger RNA encoding TNF-gamma is expressed predominantly in HUVEC cells (Figure 3B; lane 9), but not in other cell lines examined; for example; lane 1 is CAMA1 (breast cancer); lane 2 is AN3CA (uterine cancer); lane 3 is SK.UT.1 (uterine cancer); lane 4 is MG63 (osteoblastoma); lane 5 is HOS (osteoblastoma); lane 6 is MCF7 (breast cancer); lane 7 is OVCAR-3 (ovarian cancer); lane 8 is CAO V-3 (ovarian cancer); lane 10 is AOSMIC (smooth muscle); and lane 11 is foreskin fibroblast.

Northern blot analyses were also performed to determine the relative expression level of the TNF-gamma RNA with respect to the proliferation state of HUVEC cell cultures. In these experiments, identical amounts of total RNA isolated from HUVEC cells (15 µg) were electrophoretically separated and blotted as described above. RNA was isolated from cultures 1, 2, 3, 4, 6, and 7 days post-seeding. As illustrated in Figure 4, TNF-gamma RNA (labeled "VEGI") was only seen at low levels in newly seeded cultures (days 1, 2, and 3). However, expression of TNF-gamma RNA was apparent as the HUVEC cells in the cultures began to reach confluence (days 4, 6, and 7). These experiments indicate that TNF-gamma expression increases as cells in a culture or tissue begin to reach the quiescent state of non- or reduced- proliferation.

**Example 5: Ability of Recombinant TNF-gamma to inhibit WEHI 164, ABAE, and L929 cell growth, and to induce cell adhesion in HL-60 cells.**

The adherent target cells were prepared from confluent cultures by trypsinization in PBS, and non-adherent target cells were harvested from stationary

cultures and washed once with medium. Target cells were suspended at  $3 \times 10^5$  cells/ml in medium containing 10% FCS. 0.1 ml aliquots were dispensed into 96-well flat-bottomed microtiter plates containing 0.1 ml serially diluted test samples of cells (WEHI 164 and L929). Incubation was continued for 70 hours. TNF-a, TNF-b and bacterially-produced TNF-gamma were added at a 0.5  $\mu\text{g/ml}$  concentration. The cytotoxicity and proliferation activity was quantified using an MTS assay performed by the addition of 20  $\mu\text{l}$  of MTS and phenazine methosulfate (PMS) solution to each well. After a three hour incubation, the OD at 492 nm was measured by an ELISA plate reader. The  $\text{OD}_{492}$  is proportional to the number of viable cells in the wells. The percent of cytotoxicity was calculated as follows:  $\% \text{ cytotoxicity} = (100 - \text{OD}_{\text{experimental}} / \text{OD}_{\text{control}}) \times 100$ . The photographs were taken after 72 hours. As shown by Figures 7A and 8, TNF-gamma induced a morphology change which appeared as dark round cells (indicating killed cells).

In the graph of Figure 7B, the assay was performed as described above, however, increasing amounts of TNF-a, TNF-b and TNF-gamma were added to the cultures. The results indicate that TNF-gamma is a dose-dependent inhibitor of the growth of the endothelial cell line WEHI 164, but not of the fibroblast cell line L929 (Figures 8 and 9).

A truncated form of the TNF-gamma polypeptide consisting of amino acids 12-147 of the complete TNF-gamma amino acid sequence shown as SEQ ID NO:2 (designated TNF-gamma<sub>12-147</sub>) was also used to examine the effect of TNF-gamma on endothelial cell growth. Treatment of adult bovine aortic endothelial (ABAE) cells with TNF-gamma<sub>12-147</sub> resulted in nearly complete inhibition of the growth of cells in the ABAE culture, but not of cells in the breast cancer cell lines MDA-MB-435 or MDA-MB-231 (Figure 10; TNF-gamma is designated "VEGI" in this figure). Nearly complete inhibition of the growth of the endothelial cells was achieved at 10  $\mu\text{g/ml}$  TNF-gamma<sub>39-174</sub>, with a half-maximum inhibitory concentration value ( $\text{IC}_{50}$ ) of approximately 1  $\mu\text{g/ml}$  (approximately 70 nM).

To test adhesion ability of TNF-gamma, HL-60 cells were used and cell adhesion and cell-cell contact were measured by observation under the microscope and scored subjectively by two independent investigators. Figure 11 illustrates the ability of TNF-gamma to induce cell adhesion. Cultures which were not treated with TNF-gamma contained cells which had spread throughout the culture dish. However, cultures which were treated with TNF-gamma, contained cells which were clearly aggregated together.

#### ***Example 6: Measurement of Apoptosis ability of TNF-gamma***

In a first incubation step, anti-histone antibody was fixed adsorptively on the wall of a microtiter plate module. Subsequently, non-specific binding sites on the wall were saturated by treatment with incubation buffer (e.g., blocking solution). During the second incubation step, the nucleosomes contained in the WEHI 164 cell sample treated with the TNF-a, TNF-b or bacterially-produced TNF-gamma were bound via their histone components to the immobilized anti-histone antibody. In the third incubation step, anti-DNA-peroxidase (POD) reacted with the DNA component of the nucleosomes. After removal of all unbound peroxidase conjugate by a washing step, the amount of peroxidase retained in the immunocomplex was determined spectrophotometrically using the substrate ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]). Anti-histone antibody reacted with the histones H1, H2A, H2B, H3, and H4 from the sample. Anti-DNA POD antibody bound to single- and double-stranded DNA. Therefore, the ELISA allowed the detection of mono- and oligonucleosomes and may be applied to measure apoptotic cell death. The level of cell death was measured by the amount of cytoplasmic histone-associated DNA fragments which was indicated by the ratio of the absorbances observed at 405 and 490 nm ( $A_{405}/A_{490}$ ). The results of these experiments are illustrated in Figure 12 (See Boehringer mannheim Catalogue, 0990 C 93 2 1541170).

As shown in Figure 12, WEHI 164 cells were induced to undergo increasingly high levels of apoptosis, resulting in cell death, in the presence of increasing amounts of TNF-gamma. This effect was also observed in the presence of increasing amounts of the control TNF-b or in the presence of any of the analyzed levels of the control TNF-a.

#### 25 **Example 7: Receptor binding assay using TNF-gamma**

TNF-a and bacterially-produced TNF-gamma were purified by Ni-NTA affinity chromatography using the 6-His tag fused to the terminus of the recombinant proteins. 1 µg/well of either protein was added to a nickel chelate-coated 96-well plate (Xenopore Corp.) and incubated for 2 hours. After washing three times, 100 ng of human soluble TNF receptors (specifically, sTNF RI or sTNF RII) was added to each well and incubated for 2 hours. The plate was then washed three times and alkaline phosphatase-labeled polyclonal antibodies raised against either sTNF RI or sTNF RII was added in a total volume of 200 µl. An aliquot of substrate solution (200 µl) was then added to each well and the plate was incubated for an additional 2 hours. The OD was then measured using an ELISA reader (at a test wavelength of 450 nm and a correction wavelength of 590 nm). The results shown in Figure 13 illustrate that

TNF-gamma does not bind significantly to sTNF-receptors when compared to the control binding observed with TNF-a.

**Example 8: Expression via Gene Therapy**

5        Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is  
10       inverted and the chunks of tissue remain fixed to the bottom of the flask. At this time, fresh media is added (e.g., Ham's F12 media, supplemented with 10% FBS, penicillin, and streptomycin). The culture is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every 2-3 days. After an additional two weeks in culture, a monolayer of fibroblasts will have  
15       emerged. The monolayer is trypsinized and scaled into larger flasks.

      pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988)), which is flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with *Eco* RI and *Hind* III, and, subsequently, treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified using glass beads.

20       The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an *Eco* RI site and the 3' primer includes a *Hind* III site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified *Eco* RI and *Hind* III fragment are added together, in the presence of T4 DNA ligase. The  
25       resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

      The amphotropic pA317 or GP+am12 packaging cells are grown in tissue  
30       culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

35       Fresh media is added to the transduced producer cells, and, subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove

detached producer cells. This media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it may be necessary to use a retroviral vector that has a selectable marker, such as *neo* or *his*.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

#### Example 9: *In Vitro* Angiogenesis Assay

This assay was used to determine the relative ability of TNF-gamma<sub>12-147</sub> to inhibit the FGF-2-induced formation of capillary-like tubular structures in cultures of adult bovine aortic endothelial (ABAE) cells. Three-dimensional collagen gel plates (24-well) were prepared by addition of 0.5 ml chilled solution of 0.7 mg/ml of rat tail type I collagen (Becton Dickinson Labwares, Bedford, MA) to each well containing 1 x DMEM and adjusting to neutral pH with NaHCO<sub>3</sub>. After formation of collagen gel (about 1-2 mm thickness), ABAE cells were seeded at 5 x 10<sup>4</sup> cells/well. The cultures were maintained in a humidified 5% CO<sub>2</sub> incubator at 37°C in DMEM containing 10% calf serum (HyClone, Logan, UT) supplemented with L-glutamine (2 mM) until the cultures reached confluence. The medium was then replaced with fresh medium containing 20 ng/ml of FGF-2. The effect of TNF-gamma<sub>12-147</sub> as an inhibitor of FGF-2-induced formation of capillary-like tubular structures in ABAE cultures was analyzed by supplementing the culture medium with 0.1, 0.3, 1, 3, or 10 µg/ml of TNF-gamma<sub>12-147</sub>. All cultures were then maintained at 37°C for an additional 48 hours and then discontinued by fixation with cold methanol (-20°C).

The abundance of capillary-like structures formed by ABAE cells was analyzed by using a Kotron IBAS Image Analyzer assisted with a Hamamatsu C2400 video camera and a Zeiss Axioshop microscope. The abundance of the capillary-like structures were measured as percentages of the white areas over the total areas measured. As a control, the EC<sub>50</sub> value for the angiogenic factor FGF-2 to stimulate *in vitro* angiogenesis was about 5 ng/ml. As a further control, a maximum stimulatory effect was observed at 10 ng/ml of FGF-2.

As shown in Figure 14 (in which TNF-gamma is designated "VEGI"), observable inhibition of FGF-2-induced tube formation in ABAE cultures was observed by the addition of 1, 3, and 10 µg/ml of TNF-gamma<sub>12-147</sub> (labeled as VEGI). The IC<sub>50</sub> value for the inhibition of FGF-2-induced tube formation was

approximately 1 µg/ml, which was similar to that observed for the inhibition of endothelial cell growth (see Example 5).

**Example 10: Chicken Embryonic Chorioallantoic Membrane (CAM)**

**Angiogenesis Assay**

The CAM assay was carried out essentially as described by Nguyen and colleagues (*Microvasc. Res.* **47**:31-40 (1994)) and Iruela-Arispe and Dvorak (*Thromb. Haemost.* **78**:672-677 (1997)). The method is based on the growth of new capillary vessels into a collagen gel pellet placed directly on the chorioallantoic membrane (CAM). The angiogenic factors FGF-2 (100 ng) or VEGF (250 ng) were embedded in collagen gel pellets and placed in contact with the CAM. Quantification of angiogenesis in the gels was carried out 24 hours after the placement of the gel pellets by using a Nikon fluorescence microscope. The images were transferred to a Power PC 100 AV, using a CCD Sony camera. Fluorescence intensity was evaluated with NH Image 1.61 software. Fluorescence intensity for the positive controls (which contained an angiogenic factor alone) was considered as the maximum angiogenic response, and set, arbitrarily, at 100. Due to the variability of the assay, inhibition greater than 20% was considered significant.

As an experimental determination of the effect of TNF-gamma on the FGF-2- or VEGF-induced angiogenesis, bacterially-produced TNF-gamma (250 ng) was mixed with either FGF-2 (100 ng) or VEGF (250 ng) and embedded in collagen gel pellets. The pellets were then placed in contact with the CAM as described above. As shown in Figure 15 (in which TNF-gamma is designated "VEGI"), TNF-gamma markedly inhibited new capillary growth into collagen gels.

**Example 11: In Vivo Tumorigenicity Assay**

An *in vivo* analysis of the potential effect of TNF-gamma on angiogenesis was performed using a xenograft tumor model. In this experimental approach, one million human breast carcinoma cells (MDA-MB-231 or MDA-MB-435) were injected into the mammary fat pad of female nude mice either alone or mixed with chinese hamster ovary (CHO) cells transfected with TNF-gamma or CHO cells transfected only with the CHO-vector ( $5 \times 10^6$  cells per mouse). The TNF-gamma polypeptide expressed in these experiments consisted of the polypeptide shown as SEQ ID NO:2 excluding the N-terminal 22 amino acids. The N-terminal 22 amino acids of this TNF-gamma mutein were replaced by the secretory signal peptide of human interleukin-6 (Hirano, T., *et al.*, *Nature* **324**:73-76 (1986)).

Mice which were coinjected with human breast carcinoma cells and either TNF-gamma-expressing CHO cells or vector-transfected CHO cells were then randomized and tumors were measured twice weekly. The tumor size was assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. Data are presented in Figures 16A and 16B as the mean +/- standard deviation of six mice in each group.

Results presented in Figure 16A and 16B (in which TNF-gamma is designated "VEGI") illustrate the sizes of the MDA-MB-231 and MDA-MB-435, respectively, xenograft tumors (mm<sup>2</sup>) as a function of time (days postinoculation). Tumors were measured beginning on day zero and approximately at 5 day intervals through approximately the twenty-eighth day. In each case, tumors which resulted from breast carcinoma cells coinjected with TNF-gamma-expressing CHO cells (represented by the closed circles in Figures 16A and 16B) remained significantly smaller in size than those which resulted from breast carcinoma cells coinjected with vector-only CHO cells (represented by the open circles in Figures 16A and 16B).

**Example 12: Induction of NF-kB and c-Jun kinase (JNK) by TNF-gamma.**

Activation of cellular NF-kB is preceded by the phosphorylation, ubiquitination, and ultimate degradation of an endogenous NF-kB inhibitor molecule designated Ikb $\alpha$ . Degradation of the inhibitor allows the p65 subunit of NF-kB to translocate to the nucleus where it can act as a transcriptional regulator. For this reason, an electrophoretic mobility shift analysis (EMSA) is an appropriate method for analyzing activation of cellular NF-kB by treatment of cultured cells with TNF-gamma.

In these analyses cells ( $2 \times 10^6$  per ml) were treated with different concentrations (0.1-1.0  $\mu$ g/ml) of bacterially-produced TNF-gamma at 37°C for 12 hours. Nuclear extracts were then prepared from the cultured cells and EMSA was performed as is well-known in the art and essentially as described (Singh, S. and Aggarwal, B. B. *J. Biol. Chem.* **270**:10631-10636 (1995)).

Treating U-937 cells with TNF-gamma for 12 hours resulted in increase in DNA-binding by the p65 subunit of NF-kB. Peak activation of DNA-binding by p65 was observed when U-937 cells were treated with 1  $\mu$ g/ml TNF-gamma for 12 hours. However, treatment of U-937 cells with as little as 0.2  $\mu$ g/ml TNF-gamma for 12 hours resulted in an observable increase in p65 DNA-binding. TNF-gamma was observed to activate p65 DNA-binding over basal levels from 30 minutes to 18 hours after the initiation of treatment in U-937 cells.

These experiments were elaborated by determining a degradation profile for IkBa in U-937 cells in response to treatment with TNF-gamma. A time course of IkBa degradation was determined by Western blot analysis, a technique that is well-known by one of ordinary skill in the art and has been described by Singh and Aggarwal (*J. Biol. Chem.* **270**:24995-25000 (1995)). IkBa was completely degraded when U-937 cells were treated with 0.1-1.0 µg/ml TNF-gamma for 12 hours.

The cellular kinase designated c-Jun kinase (JNK) is an early event in cellular activation. The activation of JNK by TNF-gamma was analyzed as an additional method of determining cellular reaction to treatment with TNF-gamma. The JNK kinase activation assay is well-known by one of skill in the art and has been described by Derijard and colleagues (*Cell* **76**:1025-1029 (1994)). After treatment of U-937 cells with 0.1 to 3.0 µg/ml of TNF-gamma for 12 hours, the cells were harvested and assayed for JNK kinase activity. By 6 and 12 hours, JNK activity had increased 2- and 3.6-fold, respectively.

**Example 13: Effect of TNF-gamma in treating adjuvant-induced arthritis in rats.**

An analysis of the use of TNF-gamma to treat rheumatoid arthritis (RA) may be performed through the use of an adjuvant-induced arthritis (AIA) model in rats. AIA is a well-characterized and reproducible animal model of rheumatoid arthritis which is well-known to one of ordinary skill in the art (Pearson, *Ann. Rheum. Dis.* **15**:379 (1956); Pearson & Wood, *Arthritis Rheum.* **2**:440 (1959)). TNF-gamma is expected to inhibit the increase in angiogenesis or the increase in endothelial cell proliferation required to sustain the invading pannus in bone and cartilage observed in this animal model of RA. Lewis and BB rats (available from Charles River Lab, Raleigh, NC and the University of Massachusetts Medical Center, Worcester, MA) are used as the common and responsive strains for adjuvant-induced arthritis in these experiments.

Initiation of the arthritic condition is induced by the intradermal injection of 0.1 ml adjuvant (5 mg/ml) into the base of the tail. Groups of 5 to 6 rats receive either 0.1 to 1.0 mg/kg TNF-gamma or vehicle intra-articularly 20 days after the injection of adjuvant. At this timepoint, acute inflammation reaches a maximal level and chronic pannus formation will have just begun. The effect of TNF-gamma on pannus formation is analyzed radiologically once each week after day 15 following adjuvant challenge essentially as described by Taurog and colleagues (*J. Exp. Med.* **162**:962 (1985)). Briefly, rats are anesthetized with ether or chloral hydrate and positioned so that both hind limbs are X-rayed together. The X-ray films is examined blindly using



a scoring system of 0-3 for periosteal reaction, bony erosions, joint space narrowing and destruction. When there is a significant amount of joint damage in vehicle-treated rats, the animals are sacrificed. At this point, the paws are evaluated histologically for the relative degree of tissue damage and for the therapeutic effect TNF-gamma has elicited on these joints.

Finally, TNF-gamma- and vehicle-treated animals undergo a clinical evaluation twice per week to assess hind paw volume using a plethysmometer system and body weight.

- 10 ***Example 14: DR3 Ligand (TNF-gamma) is a novel anti-tumor cytokine existing in two different forms and differentially expressed in different tissues and cells.***

**Background:**

- 15 TNF (tumor necrosis factor) superfamily members play very important roles in cell activation, proliferation, differentiation, apoptosis, cytotoxicity and immune regulation. Members of TNF ligand and receptor superfamily are often overexpressed in various human cancer cells and/or activated lymphocytes, their extracellular accessibility makes them excellent potential targets for specific antitumor therapy and immunomodulating therapy. Over the past few years the list of molecules belonging to the TNF receptor and ligand superfamily has grown rapidly. The TNF ligand family of cytokines consist of over 13 type II transmembrane proteins (except TNF-b), the TNF receptor superfamily consist of over 18 type I transmembrane proteins except OPG, also known as OCIF or TR1, which is a secreted protein, and TRID/DcR1/TRIAL-R3, which is a GPI-linked cell surface molecule.

- 25 Several TNF receptor superfamily members as well as some of the intracellular signal transducers involved in apoptosis contain a stretch of amino acids, approximately 60 to 80 amino acid long, referred to as the "death domain". These death domain-containing receptors, such as TNFR1, Fas/Apo-1/CD95, DR3 (also known as Wsl, Apo3, TRAMP or LARD), DR4, DR5 or TRAIL-R2, upon activation by their ligands, recruit various proteins that mediate cell death through the death domain. These proteins in turn recruit other proteins via their death domains or death effector domains to transduce the death signal. TNFR1 is expressed in most tissues and cell types and is involved in transducing three major types of signals: activation of the transcription factor NF-kB, c-jun N-terminal protein kinase and apoptosis.
- 30 Whereas Fas is expressed in lymphocytes, liver, heart, lung, kidney, and ovary. In contrast, DR3 is predominantly expressed in spleen, thymus, and peripheral blood lymphocytes. The ligand for DR3 has not yet been identified. DR3 interacts with

We have identified several novel TNF receptor and ligand superfamily members using several search strategies. One novel TNF-like ligand, TNF-gamma, was predominantly expressed in endothelial cells. Although TNF-gamma shares some of the activities TNF, it does not bind to TNFR1 and TNFR2, indicating that TNF-gamma binds to a distinct receptor. Here we show that TNF-gamma binds to DR3 in several receptor-ligand binding assays. Interestingly, TNF-gamma exists in two different forms which are differentially expressed in different cells and tissues.

We have identified several novel TNF receptor and ligand superfamily members from HGS database which contains over 1.5 million ESTs from over 620 cDNA libraries. One novel TNF-like ligand predominantly expressed in an endothelial cell library exhibited 20-30% sequence homology to other members of the TNF family. The protein was named TNF-gamma-alpha (or VEGIa for Vascular Endothelial derived tumor Growth Inhibitor alpha). Subsequent database analysis and library screening identified a novel splicing variant of TNF-gamma-alpha, designated TNF-gamma-beta (or VEGIb). This isoform was found predominantly in cDNA libraries of TNF $\alpha$ - and IL-1-induced endothelial cells, monocyte and activated T-cells. The cDNA for TNF-gamma-alpha encodes 174 amino acid residues and TNF-gamma-beta encodes 251 amino acids. Both proteins have characteristics of type II transmembrane proteins. They only differ at the N-terminus which corresponds to the intracellular and transmembrane domains (Figure 18A-D and 19).

Recombinant TNF-gamma induces apoptosis in several cell lines such as bovine pulmonary artery endothelial cells and adult bovine aortic endothelial cells. [Bovine pulmonary artery endothelial cells were incubated with various concentrations of TNF-gamma for 48 hours. The apoptosis was assessed by nuclear staining with Hoechst 33342 fluorescence dye (10 mg/ml).] TNF-gamma also induces nuclear factor  $\kappa$ B (NF- $\kappa$ B) and c-Jun N-terminal kinase (JNK) activation, inhibits angiogenesis *in vitro*. [U937 cells were transfected using lipofectamine (following manufacturers instruction) with 0.2 mg of reporter plasmid (NF- $\kappa$ B-SEAP). The transfected U937 cells were collected and added to the 96-well plate (200  $\mu$ l/well) with various concentrated of TNF-gamma. After Incubation at 37°C for 72 hr, the NF- $\kappa$ B activity was measured with luminometer at absorbance of 450 nm.]

To identify the novel receptor and ligand pairs, several receptor-ligand binding assays were established. Recombinant soluble TNF-gamma containing the entire ectodomain binds to DR3-Fc fusion protein immobilized on BIAcore chip, purified DR3-Fc also binds to BIAcore chip immobilized with TNF-gamma. [Purified DR3-Fc or TNF-gamma was analyzed on a BIAcore instrument flowcell derivatized with TNF-gamma or DR3-Fc. The shown data represents the net bound (off-rate) region of the plot after binding of TNF-gamma to immobilized DR3-Fc receptor, or binding of DR3-Fc to immobilized TNF-gamma, which is measured in relative mass units (RU) versus time. The binding conditions were performed at high receptor chip densities under diffusion-limited conditions.] Using immunoprecipitation techniques, recombinant TNF-gamma was co-immunoprecipitated by DR3-Fc, but not LTbR-Fc immunoadhesins. [The Fc-extracellular domains of DR3 or Fc alone and the corresponding ligands were prepared and binding assays were performed as described elsewhere. The respective Fc-fusions were precipitated with protein G-Sepharose and co-precipitated soluble ligands were detected by immunoblotting with anti-TNF-gamma antibody. Blotting and detection was performed as described in BM Chemiluminescence Western Blotting kit protocol.]

To further demonstrate the interaction between DR3 and TNF-gamma, we screened several cell lines for cell surface expression of TNF-gamma using polyclonal antibody to recombinant soluble TNF-gamma. Consistent with the Northern blot analysis, peripheral blood mononuclear cells (PBMC) and human umbilical vein endothelial cells (HUVEC) express TNF-gamma on the cell surface by immunostaining with antibody to TNF-gamma. [Cells were collected by trypsinization or aspiration, and centrifuged at 1500-2000 rpm for 5 min. The cell pellets were resuspended and washed in 5 ml ice-cold PBS twice. The cells were incubated for 30 min at 40°C with antibody (10 mg/ml) to TNF-gamma to detect expression of TNF-gamma on cell surface, with DR3-Fc or LTbR-Fc (10 mg/ml) for receptor and ligand binding in the binding buffer (HBSS containing 10% BSA, 20 mM HEPES, pH 7.2, 0.02% NaN<sub>3</sub>). Purified human IgG (25 mg/ml) was used as control. Cells were then washed and stained with phycoerythrin (PE) conjugated to goat anti-rabbit or anti-human IgG at 20 mg/ml. Fluorescence was analyzed by a FACscan flow cytometer (Becton Dickinson, Mountain View, CA).] Two tumor cell lines (MC-38/TNF-gamma and MDA-231/TNF-gamma) transfected with TNF-gamma also express TNF-gamma on the cell surface. FACS analysis showed that there is a shift in the most population following exposure MC-38/TNF-gamma cells to DR3-Fc, indicating cell-surface binding between TNF-gamma and DR3. Similarly, a shift in the MDA-231 cells transfected with TNF-gamma was observed. In addition, DR3-Fc

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It is estimated that 10% of genes can be alternatively spliced, but in many cases the function of proteins produced remains obscure. To examine the potential functional significance of the two splicing variants of TNF-gamma, PCR analysis was performed in over 100 cDNA libraries. These results are shown in the following table:

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### Differential expression pattern of DR3, TNF-gamma-alpha, and TNF-gamma-beta

5	Library	DR3	TNF-ga	TNF-gb	Library	DR3	TNF-ga	TNF-gb
	<b>Normal Tissue</b>				<b>Abnormal tissue and cell</b>			
	Liver	+		+	Hepatocellular tumor	+		
	Lymph node	+		+	Hodgkin's lymphoma	+		
10	Tonsil	+			Rhabdomyosarcoma			+
	Bone marrow	+			Nasal polyps			
	Spleen	+			Spleen, metastatic melanoma			
	Heart	+			Spleen, chronic			
	Thymus	+		+	lymphocytic leukemia			
15	Pericardium	+			Healing wound ( skin )	+		+
	Brain	+			B-cell lymphoma			
	Lung			+	Hemangiopericytoma			
	Skeletal muscle				Pancreas tumor	+		
	Placenta			+	Burned skin	+		
20	Prostate			+	Prostate cancer, stage C			
	Pituitary				U937 cell	+		
	Testis	+		+	Ovarian tumor			+
	Colon				Colon cancer, metasticized	+		+
	Pancreas	+			to liver			
25	Kidney			+	Colon Cancer			
	Kidney cortex		+		Crohn's disease			
	Pualmonary				Rejected kidney	+		+
	Adipose	+	+		T-cell lymphoma			+
	Ovary	+		+	Ovary tumor			
30	Cerebellum				Endometrial tumor			
	Hippocampus				Skin tumor			
	Hyperthalamus				Pancreatic carcinoma		+	
	Olfactory epithelium		+	+	Jurkat cells			+
	Striatum depression		+		Hela cell line	+		+
35	Pineal gland				LNCAP+0.3 nM androgen			+
					LNCAP+30 nM androgen	+		+

Fetal tissue				Normal cell			
	8 week embryo	+	+	HUVEC.	+	+	+
	9 week embryo	+		Dermal endothelial,			+
	Fetal brain	+	+	Resting T cell			
5	Fetal kidney	+	+	Activated T cell (12 hr)			
	Fetal heart	+	+	Activated T cell (16 hr)	+		
	Fetal thymus	+		Activated T cell (24 hr)	+		+
	Fetal lung	+	+	T cell helper I			
	Fetal liver	+		T cell helper II	+		
10	Fetal spleen	+		CD34+			+
				Primary dendritic cells,	+		
				Eosinophils			
				Monocytes	+		+
				Osteoblasts			
15				Keratinocyte	+		+
				Stromal endometrial cells			
				Stromal cell TF274			

As shown in the table, DR3 and two forms of TNF-gamma are differentially expressed in different tissues and cells. In the libraries tested, DR3 was found to be expressed in most tissues, in activated T-cells, monocytes, dendritic cells, TH2 cells, and several other cell lines (such as U937, HeLa) and tumor tissues (such as hepatocellular tumor and Hodgkin's lymphoma). DR3 expression was increased in LNCAP prostate carcinoma cell line treated with 30 nM of synthetic androgen. TNF-gamma-alpha is only expressed in a few tissues or cells such as fetal brain, fetal heart, adipose, kidney cortex, olfactory epithelium, pancreatic carcinoma and HUVEC. In contrast, TNF-gamma-beta has a much broader expression pattern. At the cellular level, only endothelial cell, activated T-cells, monocytes, keratinocytes, HeLa and Jurkat cells express TNF-gamma-beta. Only HUVEC, fetal brain, and fetal heart cDNA libraries express both forms of TNF-gamma and DR3. TNF-gamma-alpha, TNF-gamma-beta, and DR3 are not expressed in resting T-cells or early stage of activated T-cells (12 hr). DR3 becomes detectable at 16 hr, and both DR3 and TNF-gamma-beta become detectable in T-cells at 24 hr after PHA stimulation. The time-dependent induction of DR3 and then TNF-gamma-beta in activated T-cells suggest that DR3 and TNF-gamma may play an important role in activation induced apoptosis.

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### Conclusion:

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## Materials and methods:

### *Apoptosis assay:*

Bovine pulmonary artery endothelia cells (BPAEC) were incubated with various concentrations of TNF-gamma for 48 hours. Apoptosis was assessed morphologically and by nuclear staining with Hoechst 33342 fluorescence dye (10 mg/ml) in triplicate. Live and apoptotic cells were scored in four random fields, about 1,000 cells were counted. The DNA fragmentation was analysed as described previously.

### 10 *BIAcore receptor-ligand binding assay*

Generation of recombinant receptor DR3-Fc fusion protein and recombinant TNF-gamma were described in previous papers. Purified TNF-gamma or DR3-Fc was immobilized on BIAcore respectively. Purified DR3-Fc or TNF-gamma was analyzed on a BIAcore instrument flowcell derivatized with TNF-gamma or DR3-Fc. The net bound (off-rate) region of the plot after binding of TNF-gamma to immobilized DR3-Fc receptor, or binding of DR3-Fc to immobilized TNF-gamma, was measured in relative mass units (RU) versus time. The binding conditions were performed at high receptor chip densities under diffusion-limited conditions.

### 20 *Co-Immunoprecipitation and Western blot analysis*

Polyclonal antisera against TNF-gamma were prepared in rabbits as described previously (Ni, J., *et al.*, *J. Biol. Chem.* **272**:10853-10858, (1997)). The Fc-extracellular domains of DR3 or Fc alone and the corresponding ligands were prepared and binding assays were performed as described elsewhere. The respective Fc-fusions were precipitated with protein G-Sepharose and co-precipitated soluble ligands were detected by immunoblotting with anti-TNF-gamma antibody. The samples were loaded into a gel [NOVEX Pre-Cast Gels] (4~20% Tris-Glycine Gel). Blotting and detection was performed as described in BM Chemiluminescence Western Blotting kit protocol.

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### *FACS analysis*

Cells were collected by trypsinization or aspiration, and centrifuged at 1500-2000 rpm for 5 min. The cell pellets were resuspended and washed in 5 ml ice-cold PBS twice. The cells were incubated for 30 min at 40°C with antibody (10 mg/ml) to TNF-gamma to detect expression of TNF-gamma on cell surface, with DR3-Fc or LTbR-Fc (10 mg/ml) for receptor and ligand binding in the binding buffer (HBSS containing 10% BSA, 20 mM HEPES, pH 7.2, 0.02% NaN<sub>3</sub>). Purified

human IgG (25 mg/ml) was used as a control. Cells were then washed and stained with phycoerythrin (PE) conjugated to goat anti-rabbit or anti-human IgG at 20 mg/ml. Fluorescence was analyzed by a FACscan flow cytometer (Becton Dickinson, Mountain View, CA).

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*NF- $\kappa$ B-SEAP (secreted alkaline phosphatase) reporter assay*

U937 cells were transfected using lipofectamine (following manufacturer's instructions) with 0.2 mg of reporter plasmid (NF- $\kappa$ B-SEAP). The transfected U937 cells were collected and added to the 96-well plate (200  $\mu$ l/well) with various concentration of active TNF- $\gamma$  or inactivated (boiled) TNF- $\gamma$  or in combination with various concentration DR3-Fc receptor and 100 ng/ml of TNF- $\gamma$ . After Incubation at 37°C for 72 hr, the NF- $\kappa$ B activity was measured with luminometer at absorbance of 450 nm.

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15 *Tissue and cell distribution analysis using PCR on a large collection of cDNA libraries and cDNA database:*

To study the tissue distribution of DR3, TNF- $\gamma$ - $\alpha$  and TNF- $\gamma$ - $\beta$ , two gene specific primers were synthesized for each gene. Over 100 cDNA libraries are tested and the libraries gave a positive predicted size signal are indicated as +.

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*In vivo tumorigenicity assay:*

The full length TNF- $\gamma$  and extracellular domain of DR3 was cloned into pcDNA3 expression vector (Invitrogen, Carlsbad, CA) and transfected to MCA 38 cells, respectively. Subsequent to transfection, G418 selection, and cloning, three clones from each constructs were picked for tumorigenicity study. The expression of TNF- $\gamma$  and DR3 in MCA 38 cells were confirmed by Northern analysis. MCA 38 cells ( $1 \times 10^6$  cells/mouse) expressing TNF- $\gamma$  or DR3 extracellular domain were injected into C57BL/6 mice. Mice then were randomized and tumors were measured twice weekly. The tumor size was assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. Data are represented as the mean  $\pm$  SD of 6 mice in each group.

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35 *Example 15: TNF- $\gamma$ - $\alpha$ , a novel member of TNF cytokine family, causes endothelial cell apoptosis.*

TNF-gamma-alpha is a novel protein with a molecular weight of 22 kD that was recently identified by searching the Human Genome Sciences (HGS) cDNA database (Tan, K. B., *et al*, *Gene* **204**:35-46 (1997)). TNF-gamma-alpha is a type II membrane protein and exhibits about 30% sequence homology to human tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). This newly identified member of the TNF family has been demonstrated to be abundantly expressed in endothelial cells as well as in kidney, lung and prostate. TNF-gamma-alpha expression in HL-60 and THP1 cells was induced by PMA treatment. Radiation hybrid mapping localized TNF-gamma gene on chromosome 9q32, near CD30L. Because of its overexpression in endothelial cells, TNF-gamma-alpha has been suggested to possibly play a role in vascular functions (Tan, K. B., *et al*, *Gene* **204**:35-46). The present study was undertaken to explore whether TNF-gamma-alpha induces endothelial cell apoptosis, a phenomenon suggested to be one cause of endothelial cell damage contributing to various inflammatory disorders and cardiovascular dysfunction (Bryant, D., *et al*, *Circulation* **97**:1375-1381 (1998)). To examine this possibility, we used bovine pulmonary artery endothelial cells (BPAEC) to which TNF $\alpha$ -induced apoptosis has been demonstrated (Polunovsky, V. A., *et al*, *Exp. Cell Res.* **214**:584-594 (1994)). Apoptosis was detected on the basis of morphological (including ultrastructural) and biochemical characteristics (DNA fragmentation). In addition, we studied the effects of TNF-gamma-alpha on the activity of stress kinases, stress-activated protein kinase (SAPK/JNK) and p38 mitogen-activated protein kinase (p38 MAPK), and the caspases. Both signaling pathways are believed to be implicated in programmed cell death (Xia, Z., *et al*, *Science* **270**:1326-1331 (1995)). The expression of Fas and Bcl-2 in TNF-gamma-alpha-stimulated BPAEC was also determined in view of the death-promoting effect of Fas and the anti-apoptotic effect of Bcl-2 (Nagata, S. and Golstein, P. *Science* **267**:1449-1456 (1995)).

### Materials.

TNF-gamma-alpha protein (22 kD) was provided by HGS. Ac-YVAD-AMC and Ac-DEVD-AMC were purchased from American Peptide (Sunnyvale, CA, USA). ZVAD-fmk and Ac-YVAD-CHO were obtained from Enzyme Systems (Dublin, CA, USA) and Peptides International (Louisville, KY, USA), respectively. Ac-DQMD-AMC, Ac-LEED-AMC, Ac-VETD-AMC and anti-p38 MAPK mAb were provided by SmithKline Beecham (SB) Pharmaceuticals (King of Prussia, PA, USA). Ac-IETD-AMC and mouse-anti-human JNK mAb were purchased from Biomol

## 5 Cell Cultures.

### Morphological Assessment and Quantification of Apoptosis.

### DNA Fragmentation Analysis.

(b) *In situ* end-labeling (TUNEL): BPAEC were cultured in two-chamber slides (Nunc) and treated with TNF-gamma-alpha for 8 to 24 h. *In situ* detection of apoptotic cells was performed by using terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling with an ApopTag *in situ* apoptosis detection kit (Oncor) following the manufacturer's recommendation.

#### *Stress-activated Protein Kinase (SAJPK/JNK) Assay.*

SAPK activity was measured using GST-c-Jun<sub>(1-81)</sub> as bound to glutathione-Sepharose 4B as described previously (Yuc, T. L., *et al.*, *Mol. Pharmacol.* **51**:951-962 (1997)). Briefly, the cells were treated with vehicle or TNF-gamma-alpha, washed, and lysed in lysis buffer. The nuclear-free supernatant was normalized for protein content and immuno-precipitated with anti-SAPK antibody-conjugated Sepharose beads. The mixture was rotated 4°C for 3 h. The phosphorylation buffer containing GST-c-Jun<sub>(1-81)</sub>, 10 µCi [ $\gamma$ -<sup>32</sup>P] ATP, 125 µM ATP and 100 mM MgCl<sub>2</sub> was added to the SAPK-bound beads in assay buffer. The reaction was terminated after 20 min at 30°C by addition of protein loading buffer and heated at 90°C for 3 min. Phosphorylated proteins were resolved in 10% SDA-polyacrylamide gel electrophoresis followed by autoradiography. The intensity of the bands was quantified by PhosphorImager (Yuc, T. L., *et al.*, *J. Mol. Cell. Cardiol.* **30**:495-507 (1998)).

#### 15 *p38 MAPK Assay.*

The cell lysates prepared as above were immuno-precipitated with anti-p38 MAPK antibody bound to protein A agarose for 4 h at 4°C. The beads were washed with lysis buffer and then with kinase buffer as described previously (Kumar, S. M., *et al.*, *J. Biol. Chem.* **271**:30864-30869 (1996)). The immune-complex kinase assay was initiated by the addition of 25 µl of kinase buffer containing 2 µg of GST-ATF2 and 50 µM [ $\gamma$ -<sup>32</sup>P] ATP (20 Ci/mmol). The phosphorylated products were resolved by SDA-PAGE and visualized by Phosphorimage.

#### *In Vitro Transfection of Dominant-interfering Mutant of c-JUN in BPAEC.*

25 The cells were plated in two-chamber slides. The cells were cotransfected with 0.5 µg/ml of Pegfp-c-1 (Clontech; Li, Y. and Horwitz, M. S. *Biotechnology* **23**:1026-1028) as a fluorescent marker of transfected cells together with 1 µg/ml of either the empty cloning vector pCDNA1 (control) or the dominant-interfering c-Jun mutant pcDNA1-FigΔ169 (Xia, Z., *et al.*, *Science* **270**:1326-1331 (1995)) using Calphos Maximizer Transfection Kit (Clontech) according to the manufacturer's recommendation. Following transfection, the cells were allowed to recover in complete medium for 24 h. The cells were treated with TNF-gamma-alpha and the number of apoptotic cells was assessed by nuclear staining after fixation as described in Methods.

### *Caspase Activity Assay.*

The cells were treated with vehicle of TNF-gamma-alpha. Caspase activity assays were performed as reported previously (Yuc, T. L., *et al.*, *supra*). Briefly, cells were harvested and suspended in buffer containing 25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mM DDT, 5 mM PMSF, and 1  $\mu$ M pepstatin A. The suspension was forced through a 25 gauge needle 10 times to break cells. the homogenate was centrifuged at 100,000 x g for 1 h, and the cleared lysates were used for enzyme assays. Cell extracts (5-20  $\mu$ g protein) were diluted into the assay buffer (Table 2) and preincubated for 10 min at 30 °C prior to the addition of the substrates. Levels of released 7-amino-4-methylcoumarin (AMC) were measured with a Cytofluor-4000 fluorescent plate reader (Perseptive Biosystems) at an excitation and emission wavelengths of 360 nm and 460 nm, respectively.

### *Immunohistochemical Analysis for Fas, Bcl-2 and Caspase-3 Expression.*

The cells were cultured in two-chamber slides. After treatment with vehicle or TNF-gamma-alpha, the cells were fixed with 4% paraformaldehyde for 30 min at 4°C and then changed to cold PBS. The cells were treated with 0.2% Triton X-100 for 40 min at 4°C, washed with cold PBS and then non-specific immunoglobulin binding sites were blocked with normal goat serum (Vector Laboratories) for 1 h at room temperature. The cell samples were incubated with the primary antibody mouse anti-human Fas (Upstate Biotechnology), mouse anti-human Bcl-2 (DAKO) or rabbit anti-human CPP32 p17 peptide polyclonal antisera (SmithKline Beecham), for 1 h at room temperature. As a negative control, the cell samples were incubated with nonimmune IgG (for Bcl-2 and CPP32) or IgM (for Fas) instead of the primary antibody. After incubation with the primary antibody, cells were washed with PBS and then incubated for 30 min with a secondary antibody conjugated to fluorescein isothiocyanate. Cells were washed, treated with Vectashield mounting medium (Vector Laboratories) and viewed by fluorescence microscopy (Olympus IX70).

### *Statistical Analysis.*

All values are represented as mean  $\pm$  S.E.M. of n independent experiments. Statistical evaluation was performed by using one-way analysis of variance. Differences with a value of  $p < 0.05$  were considered significant.

## **RESULTS:**

### *TNF-gamma-alpha Induces Apoptosis in BPAEC.*

When BPAEC were exposed to TNF-gamma-alpha the cells shrunk and retracted from their neighboring cells, and the cytoplasm became condensed. Cells

stained with Hoechst 33324 and assessed by fluorescence microscopy demonstrated condensed chromatin of fragmented nuclei and blebbing of the plasma membrane. The study with transmission electron microscopy showed that TNF-gamma-alpha-treated BPAEC contained many cells undergoing morphologic alterations characteristic of apoptosis including condensation of chromatin and appearance of apoptotic bodies. The characteristic degradation of DNA into oligonucleosomal-length fragmentation was observed when the cells were exposed to TNF-gamma-alpha (30-300 ng/ml) for 24 h. The DNA fragments *in situ* was further visualized by using TUNEL method. A considerable fraction of endothelial cells treated with TNF-gamma-alpha showed positive staining; no positively stained cells were found in the vehicle-treated cultures.

TNF-gamma-alpha-induced endothelial cell apoptosis was a time- and concentration-dependent process with an  $EC_{50}$  value of 72 ng/ml. A significant increase in the number of cells with apoptotic morphological changes was apparent 6-8 h after exposure of the cells to TNF-gamma-alpha. Under similar conditions, TNF-a at 10 ng/ml induced apoptosis in PEAPC by  $16.7 \pm 3.2\%$  (n=4).

#### *Effects of sTNFR1 and sTNFR2 on TNF-gamma-alpha-induced apoptosis in BPAEC.*

Neither sTNFR1 nor sTNFR2 showed effect on TNF-gamma-alpha-induced apoptosis in BPAEC. Under the same condition TNFa-induced apoptosis in BPAHC was reduced by sTNFR1 significantly.

Regulation of Fas and Bcl-2 Expression in Endothelial Cells by TNF-gamma-alpha. Immunocytochemical analysis of Fas and Bcl-2 proteins was determined at 8 and 24 h after treatment with TNF-gamma-alpha. The basal level of Fas in BPAEC was undetectable. However, a significant number of cells expressing Fas receptor were detected at 8 and 24 h after stimulation. When mouse IgM was substituted for the primary antibody, positive Fas immunoreactivity was not detected. In contrast, Bcl-2 expression was not detected in neither unstimulated nor TNF-gamma-alpha-treated BPAEC.

#### *Activation of SAPK/JNK and p-38 MAPK.*

With regard to the effects of TNF-gamma-alpha on SAPK/JNK activity in BPAEC, exposure of endothelial cells to TNF-gamma-alpha induced a rapid activation of SAPK/JNK. A significant increase in SAPK/JNK activity was detected 20 min after stimulation, peaked at 40 min. and then returned to the basal levels after 60 min. TNF-gamma-alpha-induced activation of SAPK/JNK in endothelial cells is a concentration-dependent process. Some basal activities of SAPK/JNK activity was

increased by  $5.6 \pm 1.4$  folds ( $p < 0.05$   $n=4$ ) and  $9.1 \pm 1.8$  folds ( $p < 0.01$   $n=6$ ) over the basal level in the presence of 50 and 300 ng/ml of TNF-gamma-alpha, respectively. TNF-gamma-alpha activated p38 MAPK in BPAEC with a similar time course as SAPK/JNK but to a lesser extent. The peak of p38 MAPK activity was increased by  $3.1 \pm 0.5$  and  $3.8 \pm 0.4$  folds over the basal level in the presence of 100 and 300 ng/ml of TNF-gamma-alpha, respectively.

*Effects on TNF-gamma-alpha-induced Apoptosis by Expression of Dominant-interfering mutant of c-JUN in BPAEC or by the p38 MAPK inhibitor, SB203580.*

To investigate the role of SAPK/JNK in TNF-gamma-alpha-induced apoptosis in BPAEC, we transfected BPAEC with a dominant-interfering mutant of c-JUN, pCDNA1-Flag $\Delta$ 169, in which a deletion in the NH<sub>2</sub>-terminal transactivation domain that includes the binding site for JNK (Xia, Z., *et al.*, *supra*). Expression of dominant-interfering c-JUN construct in BPAEC reduced TNF-gamma-alpha-induced apoptosis by 62.8% ( $p < 0.05$ ). TNF-gamma-alpha-induced apoptosis in BPAEC was also attenuated by a specific p38 MAPK inhibitor, SB203580, in a concentration dependent manner. In the presence of 3 and 10  $\mu$ M of SB203580, TNF-gamma-alpha-induced BPAEC apoptosis was reduced by 33% ( $p < 0.05$ ) and 51% ( $p < 0.01$ ), respectively. No further inhibition was observed when the concentration of SB203580 was increased.

*Activation of Caspases in BPAEC by TNF-gamma-alpha.*

TNF-gamma-alpha-induced BPAEC apoptosis was attenuated by ZVAD-fmk, an irreversible cell-permeable inhibitor of caspase (Jacobson, N. L., *et al.*, *Cell Biol.* **133**:1041-1051 (1996)), added to the culture medium 1 h prior to TNF-gamma-alpha treatment. Under the same conditions, the addition of Ac-YYAD-CHO, a relatively specific inhibitor of caspase-1 (Thorberry, N. A., *et al.*, *Nature (Lond)* **356**:768-774 (1992)), up to 100  $\mu$ M showed no effect in enhancing BPAEC rescue. To further determine which of the caspase family members are activated in the TNF-gamma-alpha-induced apoptotic process in the endothelial cells, we examined cell extracts for proteolytic activity. The relative rates of AMC formation were measured with a series of defined peptide sequence variants that are relatively specific for caspase 1, 3, 4, 7, or 8 under the optimal conditions as described previously (Yuc, T. L., *et al.*, *supra*). Similar results were observed from three repeated experiments. Cell extracts from TNF-gamma-alpha-treated BPAEC were highly active on Ac-DEVD-AMC and to a lesser extent on Ac-DQMD-AMC, but not active on the remaining three substrates which are more specific for caspase 1, 4, and 8. The



proteolytic activity appeared at 6 h after the cells were treated with TNF-gamma-alpha, peaked at 24 h, and gradually returned to basal levels within 48h. The relative velocities of four substrate hydrolysis rates by the TNF-gamma-alpha-treated cell extracts and recombinant caspase-3 were compared. The relative velocities of the two enzyme sources of four substrates were very similar.

To further confirm that caspase-3 is activated by TNF-gamma-alpha in BPAEC, immunocytochemical detection of its enzymatically active form, the 17-kD subunit, was performed. The antibody was raised against a peptide from the C-terminal portion of the p17 subunit. The neoepitope antibody only binds caspase-3 if there has been specific cleavage between the "p-10" and "p-20" subunits. Using this neoepitope antibody, only processed caspase-3 is detected, but not the proenzyme (Yuc, T. L., *et al.*, *supra*). The 17 kD subunit of caspase-3 was detected in TNF-gamma-alpha-treated but not vehicle-treated BPAEC, and was localized with fragmented nuclei within the cells.

## DISCUSSION:

The studies presented in this paper demonstrate that TNF-gamma-alpha, a novel TNF-like cytokine and a type II transmembrane protein, induces intensive apoptosis in cultured endothelial cells as reflected by morphological and biochemical criteria. Under our experimental conditions, spontaneous BPAEC death was approximately 2-4% which is in accord with a previous observation (Polunovsky, V. A., *et al.*, *supra*). The effect of TNF-gamma-alpha was concentration-dependent with an  $EC_{50}$  value of 72 ng/ml (3.5 nM) and a significant number of apoptotic cells was detected 6-8 h after treatment. Moreover, the expression of pro-apoptotic gene, Fas, was demonstrated in TNF-gamma-alpha-treated BPAEC, which is consistent with that observed in apoptotic endothelial cells reported previously (Yuc, T. L., *et al.*, *supra*).

The receptor(s) mediating TNF-gamma-alpha activity has not been identified as yet. To examine whether TNF-gamma-alpha acts via distinct receptor(s), we tested the effects of sTNFR1 and sTNFR2 on TNF-gamma-alpha-induced apoptosis in BPAEC. These two TNFRs have been shown previously to block the cell surface TNFR1 and TNFR2 mediated TNF bioactivities on responsive cell lines (data from R&D Systems). Neither sTNFR1 nor sTNFR2 inhibited the effect of TNF-gamma-alpha on BPAEC. In contrast, TNFa-induced apoptosis in BPAEC was significantly reduced by sTNF1. The results suggest clearly that TNF-gamma-alpha-induced cell death is independent of sTNFR1 or TNFR2.

Recent research efforts on TNF family members have demonstrated that TNFa and Fas activate stress protein kinases, SAPK/JNK and p38 MAPK, in a variety of

cell types (Sluss, H. K., *et al.*, *Cell Biol.* **14**:8376-8384 (1994)), however, the effects of other members of this family on SAPK and p38 MAPK are not well studies. Moreover, controversies regarding the role of SAPK/JNK and p38 MAPK in TNF $\alpha$  or Fas-mediated cell death have been reported. For example, TNF $\alpha$ -induced apoptosis is dependent on JNK activity in U937 cells (Verjeij, M., *et al.*, *Nature (Lond)* **380**:75-79 (1995); Zanke, B. W., *et al.*, *Curr. Biol.* **6**:606-613 (1996)) but not in fibroblasts (Reinhard, C., *et al.*, *EMBO J.* **16**:1080-1092 (1997)) indicating that the consequences of JNK activation vary considerably among cell types. Fas-mediated JNK activation occurs with a different kinetics from that of TNF $\alpha$ , suggesting that TNF $\alpha$  and Fas most likely activate JNK through a different mechanism (Wilson, D. J., *et al.*, *Eur. J. Immunol.* **26**:989-994 (1996)). Moreover, Juo, *et al.*, reported recently that blockade of p38 MAPK by a specific p38 MAPK inhibitor did not affect Fas-mediated apoptosis in Jurkat cells (Juo, P., *et al.*, *Mol. Cell Biol.* **17**:24-35 (1997)). Therefore, we were interested in finding whether TNF-gamma-alpha activates JNK and p38 MAPK, and what is the role of this activation in TNF-gamma-alpha-mediated apoptosis in BPAEC. The present investigation clearly demonstrates that both JNK and p38 MAPK were rapidly activated by TNF-gamma-alpha in a similar fashion as observed in TNF $\alpha$ -activated U937. Moreover, expression of dominant-interfering mutant of c-JUN in BPAEC reduced TNF-gamma-alpha-induced cell death indicating that TNF-gamma-alpha-induced apoptosis in BPAEC was dependent on JNK activity. To address the potential involvement of p38 MAPK in TNF-gamma-alpha-mediated apoptosis in BPAEC, a specific p38 MAPK inhibitor SB203580 was tested. This inhibitor has been shown to specifically inhibit p38 MAPK activity in vitro with no effect on a variety of kinases tested, including JNK and ERK-1 (Cuenda, A., *et al.*, *FEBS Lett.* **364**:229-233 (1995)). TNF-gamma-alpha-induced apoptosis in BPAEC was also reduced by SB203580 in a concentration-dependent manner, indicating that p38 MAPK signaling pathway is involved in TNF-gamma-alpha-mediated BPAEC apoptosis. This effect is different from that observed in Fas-mediated apoptosis in Jurkat cells in which SB203580 had no protective effect (Juo, P., *et al.*, *supra*). Moreover, TNF-gamma-alpha-induced p38 MAPK activation occurs with must faster kinetics in BPAEC than that observed in Jurkat cells in which the peak of p38 MAPK activation was at 2-4 h after stimulation by Fas, indicating TNF-gamma-alpha and Fas most likely activate p38 MAPK through a different mechanism with a different outcome. Our data further suggests that different members of TNF family may have different signaling pathways to mediate cell death or have different effects in different cell types.

Recent work has supported a central role for the caspase family members, as effectors of apoptosis (Kumar, S. M., *et al.*, *supra*). However, the role of caspases in endothelial cell apoptosis has not been sufficiently explored. Two characteristic features of the caspase family have been elucidated; they cleave their target proteins after specific aspartic acids, resulting in two subunits that together form the active site of the enzyme (Nicholson, D. W., *et al.*, *Nature (Lond)* **376**:37-43 (1995); Kumar, S. M., *et al.*, *supra*). Among the caspase family, caspase-3 (CPP32) has been considered as a central component of the proteolytic cascade during apoptosis and plays a key role in this family (Wang, X., *EMBO J.* **15**:1012-1020 (1996); Woo, M., *et al.*, *Gene Development* **12**:806-819 (1998)). TNF-gamma-alpha-induced BPAEC apoptosis was inhibited by ZVAD-fmk, indicating a potential role for the caspase family in this effector pathway for apoptosis. To determine which of the caspase family members are involved, we examined the substrate specificity of proteolytic activity in the extracts from TNF-gamma-alpha-activated BPAEC by measuring the relative rate of AMC formation from 6 different substrates which are relatively specific for caspases 1, 3, 4, 7 and 8 (Talanian, R. V., *et al.*, *J. Biol. Chem.* **272**:9677-9682 (1997)). Treatment of BPAEC with TNF-gamma-alpha resulted in a significant increase in proteolytic activity towards DEVD-AMC mainly and DQMD-AMC to some extent, both of which show the relative specificity for caspase-3 (Kumar, S. M., *et al.*, *supra*). There was no induction in proteolytic activity in TNF-gamma-alpha-activated cell extracts when Ac-YVAD-AMC, LEED-AMC or VETD-AMC were used as the substrate, indicating that caspases 1, 4 and 8 might not be involved. Moreover, comparison of the substrate specificity of the extracts from TNF-gamma-alpha-treated BPAEC with recombinant caspase-3 showed a similar pattern, further suggesting that caspase-3 may be the predominant member in the caspase family activated by TNF-gamma-alpha. Furthermore, immunocytochemical studies detected the active form of caspase-3 in TNF-gamma-alpha treated BPAEC. It was reported that multiple caspase homologues were found in both the cytoplasm and nucleus in etoposid-induced apoptosis in HL-60 cells (Martins, I. M., *et al.*, *J. Biol. Chem.* **272**:7421-7430 (1997)). Interestingly, in TNF-gamma-alpha-induced apoptotic BPAEC the immunoreactive 17kD subunit of caspase-3 was only localized with fragmented nuclei, further indicating a role of caspase-3 in TNF-gamma-alpha-induced apoptosis. Whether this active caspase-3 was transported into the nucleus or the inactive caspase-3 is already in the nucleus awaiting activation promoted by TNF-gamma-alpha requires further investigation. Taken together, these results suggest that caspase-3 was activated by TNF-gamma-alpha-induced cell apoptosis. However, our results cannot exclude other members of this family, especially those closely related to

caspase-3, such as caspase-7, in mediating TNF-gamma-alpha-induced apoptosis. Moreover, ZVAD-fmk was less effective at the later time (30 h) compared to the earlier time (14 h) for inhibiting TNF-gamma-alpha-induced apoptosis in BPAEC, suggesting a caspase-independent of negative-feedback mechanism may exist at the later phase of TNF-gamma-alpha-induced BPAEC apoptosis.

In summary, the present studies have demonstrated that TNF-gamma-alpha, a novel member of TNF cytokine family, causes endothelial cell apoptosis. TNF-gamma-alpha appears to act through a receptor which is distinct from TNF receptors 1 or 2. The effect of TNF-gamma-alpha is via activation of the stress protein kinases, SAPK/JNK and p38 MAPK., and the caspases, mainly caspase-3 like protease. Apoptotic programmed cell death has been suggested to be a cause of endothelial cell damage contributing to various inflammatory disorders and cardiovascular injury (Karsan, A. *Trends Cardiovasc. Med.* **8**:19-24 (1998)). Moreover, endothelial cell apoptosis may be an important mechanism involved in a balance between antiangiogenic and proangiogenic processes, and loss of this balance will lead to a variety of diseases such as solid tumor metastasis and retinopathy (Folkman, J. and Shing, J. *J. Biol. Chem.* **267**:10931-10934 (1992); Brooks, P. C., *et al.*, *Cell* **79**:1157-1164 (1994)).

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

Further, the Sequence Listing submitted herewith in both computer and paper forms are hereby incorporated by reference in their entireties.